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14. ABSTRACT Energy demand by contemporary societies and the excessive consumption of fossil fuels have impelled research and the employment of renewable energy systems. It has been proposed, in terms of renewable systems, the use of biofuels generated by the degradation of organic matter, like bioethanol, biodiesel and methane, being this last one the more efficient one based on its calorific value. For this reason we propose the implementation of anaerobic reactors which degrade biomass that has relatively high					
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ABSTRACT

Energy demand by contemporary societies and the excessive consumption of fossil fuels have impulsed research and the employment of renewable energy systems. It has been proposed, in terms of renewable systems, the use of biofuels generated by the degradation of organic matter, like bioethanol, biodiesel and methane, being this last one the more efficient one based on its calorific value. For this reason we propose the implementation of anaerobic reactors which degrade biomass that has relatively high growth rates, require low quantity of nutrients and eliminate any competition by its use, thus creating a cost-effective system. Tropical climbing vines provide biomasses with the previous characteristics; however, they contain high concentrations of cellulose and lignin that are polymers difficult to degrade. In contrast, biomass like marine algae contains low concentrations of both lignin and cellulose, which should make them an easier material for degradation. Finally, in comparison to marine algae another source of marine biomass, which can serve as biomass for the creation of these systems, is seagrasses. Nonetheless, seagrasses are more related to terrestrial plants than marine algae for which they could present the same difficulties towards degradation as climbing vines. This study aims to compare the efficiency of three different vegetation biomasses (marine algae, seagrass and tropical climbing vines) as primary substrate for anaerobic reactors. Moreover, to achieve what could be a highly cost effective system, the isolation and identification of anaerobic alginate degraders was studied. Alginate is a complex polysaccharide present in marine algae's cell wall, representing up to 40% of its dry weight. The study was completed creating anaerobic microcosms, which contained 0.016 g/mL (0.5 g total biomass) of each biomass. Methane and intermediaries produced were determined for each microcosm. After chemical determinations, the microbial community was analyzed using molecular techniques. The isolation of anaerobic alginate degraders was achieved performing serial dilutions for the purification of the microbial community present in the samples selected, which in turn were analyzed using molecular techniques, such as PCR and DGGE. After 108 days, results demonstrated that there were significant differences between marine and terrestrial biomasses; the latter was the most efficient. In terrestrial biomass microcosms, a maximum production of 50% of methane achieved an energetic rendition of 8.37 W/h; in comparison with marine biomass microcosms in which the highest energetic rendition was 3.60 W/h. Molecular analysis of the microbial community present in the marine biomass microcosms had a low diversity. In the different marine and terrestrial microcosms, the bacteria that dominated was *Desulfovibrio vulgaris*, but the dominant methanogen depended on the biomass being degraded. Moreover, in the samples analyzed, the anaerobic alginate degraders demonstrated a convergence of a gram-negative spore former, which seemed favored during the purification process. Nonetheless, even though the isolation of anaerobic alginate degraders was successful, it is important that the microbial community works together to achieve the conversion of alginate to methane. Although there was a positive isolation of bacteria that can degrade alginate, methanogenic bacteria were not isolated this in spite of methane formation, which indicated their presence in the experimental microcosms.

**Anaerobic degradation of marine algae, seagrass and
tropical climbing vines to produce a renewable energy
source and the analysis of their anaerobic microbial
communities**

By
Karla Marie Márquez Nogueras

A thesis submitted as partial fulfillment of the requirements for the degree of

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Abstract

Energy demand by contemporary societies and the excessive consumption of fossil fuels have impulsed research and the employment of renewable energy systems. It has been proposed, in terms of renewable systems, the use of biofuels generated by the degradation of organic matter, like bioethanol, biodiesel and methane, being this last one the more efficient one based on its calorific value. For this reason we propose the implementation of anaerobic reactors which degrade biomass that has relatively high growth rates, require low quantity of nutrients and eliminate any competition by its use, thus creating a cost-effective system. Tropical climbing vines provide biomasses with the previous characteristics; however, they contain high concentrations of cellulose and lignin that are polymers difficult to degrade. In contrast, biomass like marine algae contains low concentrations of both lignin and cellulose, which should make them an easier material for degradation. Finally, in comparison to marine algae another source of marine biomass, which can serve as biomass for the creation of these systems, is seagrasses. Nonetheless, seagrasses are more related to terrestrial plants than marine algae for which they could present the same difficulties towards degradation as climbing vines. This study aims to compare the efficiency of three different vegetation biomasses (marine algae, seagrass and tropical climbing vines) as primary substrate for anaerobic reactors. Moreover, to achieve what could be a highly cost effective system, the isolation and identification of anaerobic alginate degraders was studied. Alginate is a complex polysaccharide present in marine algae's cell wall, representing up to 40% of its dry weight. The study was completed creating anaerobic microcosms, which contained 0.016 g/mL (0.5 g total biomass) of each biomass. Methane and intermediaries produced were determined for each microcosm. After chemical determinations, the microbial community was analyzed using molecular techniques. The isolation of anaerobic alginate degraders was achieved performing serial dilutions for the purification of the microbial community present in the samples selected, which in turn were analyzed using molecular techniques, such as PCR and DGGE. After 108 days, results demonstrated that there were significant differences between marine and terrestrial biomasses; the latter was the most efficient. In terrestrial biomass microcosms, a maximum production of 50% of methane achieved an energetic rendition of 8.37 W/h; in comparison with marine biomass microcosms in which the highest energetic rendition was 3.60 W/h. Molecular analysis of the microbial community present in the marine biomass microcosms had a low diversity. In the different marine and terrestrial microcosms, the bacteria that dominated was *Desulfovibrio vulgaris*, but the dominant methanogen depended on the biomass being degraded. Moreover, in the samples analyzed, the anaerobic alginate degraders demonstrated a convergence of a gram-negative spore former, which seemed favored during the purification process. Nonetheless, even though the isolation of anaerobic alginate degraders was successful, it is important that the microbial community works together to achieve the conversion of alginate to methane. Although there was a positive isolation of bacteria that can degrade alginate, methanogenic bacteria were not isolated this in spite of methane formation, which indicated their presence in the experimental microcosms.

Resumen

La necesidad energética requerida por la sociedad y el consumo excesivo de combustible fósil ha impulsado la investigación e implementación de sistemas de energía renovable. Actualmente se ha propuesto el uso de biocombustibles generados de la degradación de materia orgánica, entre los cuales se encuentra el bioetanol, biodiesel y metano, siendo este último el de mayor eficiencia por su valor calorífico. En base a lo previo proponemos la implementación de bioreactores anaeróbicos los cuales degradarían biomásas con tasas de crecimiento rápidas, requieren bajas cantidades de nutrientes y eliminan cualquier competencia por su uso, creando así un sistema costo efectivo. Entre las biomásas que poseen estas características se encuentran las enredaderas tropicales, ya que poseen una tasa de crecimiento alta y no requieren altas cantidades de nutrientes para su crecimiento; más sin embargo éstas contienen una alta cantidad de polímeros complejos, como la lignina y celulosa, los cuales son difíciles de degradar. Por otro lado, biomásas como las algas marinas contienen bajas concentraciones de lignina y celulosa, lo cual las haría un material fácil de degradar. Por último, en comparación con las algas marinas, otra biomasa marina que podría cualificar para la utilización en estos sistemas son las yerbas marinas. Sin embargo, en comparación con las algas marinas éstas tienen mayor relación con las plantas terrestres por lo cual podrían enfrentar el problema de tener polímeros complejos. Es por ello que en esta investigación se trató de comparar la efectividad de algas marinas, yerbas marinas y enredaderas tropicales como sustrato primario en reactores anaeróbicos. Aun así, para lograr lo que sería un sistema altamente costo efectivo, se ha propuesto también aislar e identificar degradadores anaeróbicos de alginato. El alginato es un polisacárido presente en la pared celular de las algas marinas, representando hasta un 40% del peso seco de éstas. Para lograr los objetivos propuestos, microcosmos anaeróbicos que contenían 0.016 g/mL de cada biomasa fueron analizados en respecto a la producción de metano e intermediarios. Luego de esto, las comunidades microbianas presente en estos fue analizadas a través de técnicas moleculares. El aislamiento de degradadores anaeróbicos de alginato fue logrado realizando diluciones seriales para la purificación de la comunidad microbiana presente en las muestras seleccionadas. Luego de 108 días, los resultados obtenidos demuestran una diferencia significativa entre la producción de metano entre la biomasa marina y la terrestre, siendo esta última la más eficiente. Con una producción máxima de 50% de metano, se lograría obtener un rendimiento energético de 8.37 W/h, en comparación con las otras biomásas utilizada donde la mayor producción de energía obtenida es de 3.60 W/h. El análisis de la comunidad microbiana presente en las biomásas demuestra que hay una baja diversidad en los microcosmos de biomasa marina. En términos de las bacterias presente, aparenta existir una dominancia de *Desulfovibrio vulgaris* en todos lo microcosmos, mientras que la arquea dominante dependerá de la biomasa que se encuentre degradando la población. Más aún, el análisis de degradadores anaeróbicos de alginato demuestra la convergencia de un formador de esporas gram-negativo en los sedimentos analizados, el cual muestra ser favorecido durante el proceso de purificación. Sin embargo, aún al lograr el aislamiento de microorganismos degradadores de alginato en ambientes anaeróbicos, es importante que esta población trabaje en conjunto para lograr la conversión de alginato a metano. Aunque hubo una exitosa aislación de bacterias degradadoras de alginato, las arqueas metanogénicas no fueron aisladas a pesar de la presencia de estas en los microcosmos analizados.

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Dedication

To those special people who never let me surrender to my falls.

To my family, for letting me achieve all my dreams, you are the inspiration behind all my goals and dreams.

Mom, Dad, Mickey and Zuania, all my hard work are for you guys.

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Introduction

High concentrations of carbon dioxide in the atmosphere, released primarily by burning of fossil fuels, have ignited the search for alternative fuels to reduce global warming. Biofuels are referred to as solid, liquid, or gaseous fuels that are produced from renewable feedstock (Dermibas, 2011). Fuels, like bioethanol and biodiesel, have created a great predicament, which is based on the feedstock from which they are usually derived: soybean, maize, and sugarcane. These three items are part of the food industry and thus their use creates competition, whether they will be used for food or biofuel production. However, an innovative alternative for renewable energy is the product of the biologically mediated anaerobic decomposition of organic matter, which produces methane and CO_2 serves as an electron acceptor (Bryant, 1979). This valuable gaseous product is an energy carrier for power and biofuel production (Pakarine, 2008). For this reason, research is refocusing towards the use of a biomass that does not create a competition, like industrial wastes or cellulolytic biomass where the product of their anaerobic degradation is methane.

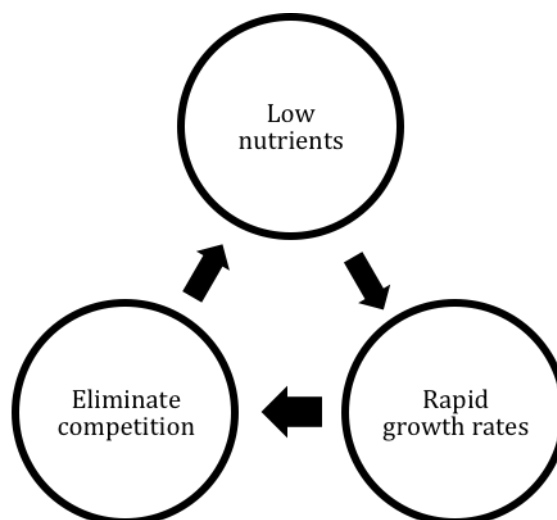


Figure 1. Ideal characteristic for biomass selection as primary substrate for the creation of a renewable energy system.

Climbing vines have evoked little interest in scientists for their study. Nevertheless their biomass could be used as feedstock for the creation of second-generation biofuels; this biofuel refers largely to lignocellulosic materials (Naik, 2010). It has been reported that vines can reduce the growth rate and productivity of trees, lower forest renewal rates, slow trees regeneration, and reduce the carbon sequestration in forest biomass (Haitan, 2011). Even though climbing vines are generally discarded because of their negative impact in ecosystems, they could have a positive outcome being used as biomass for biofuel production. The main problem that could be present in the degradation of such biomass is their structural composition rich in cellulose, lignin and hemicellulose, and the different requirements to degrade the selected biomass. In contrast to climbing vines another biomass that could be a future prospect for biofuel creation are marine macro-algae, which are currently used to produce biodiesel (Hossain, 2008).

Algae are a varied assemblage of photosynthetic eukaryotes, essentially all of those that are not classified as land plants (Lewin, 1976). Marine algae contain high concentrations of polysaccharides (alginate, laminarin and mannitol) with low lignin and cellulose content; therefore they could be an easy material to convert to methane by anaerobic digestion (Vergara-Fernández, 2008). Based on the conversion single units of sugars that can be incorporated as part of any metabolism, these complex sugars present in algae are relatively easy to break down (Anastasakis, 2011). A comparative study of which biomass is a better substrate for the anaerobic

bioconversion to methane could give a possible insight for the selection of an efficient and competitive biomass for the creation of a resourceful biofuel.

Moreover, alginate is one of the main components on the cell wall of algae, and the description of anaerobic microorganisms capable of its degradation is scarce at best. The polymers present in alginate are linear copolymers of d-mannuronic and l-guluronic acid (Gomez, 2009). The rupture of these bonds could represent a high-energy release, and can be used as precursor structure for an anaerobic degradation and consequently methane production. The description of these microorganisms could be an advantage in the anoxic degradation of marine algae. The addition of these microorganisms could speed the process of degradation and could provide a higher yield of methane production.

In addition to marine algae, another aquatic biomass which could be analyzed for anaerobic digestion could be seagrasses. An important characteristic of seagrass is the fact that they have the ability to adapt to extreme changes in their environment (de Boer, 2007), and are one of the most dynamic and productive environments in marine habitats (Lee, 2007). In comparison to marine algae, growth rates will depend on various biological and physical factors; however, it has been documented that average production of some species is near 0.816 kg/m^2 in a year (Duarte, 1999). Nevertheless, no study has been performed to determine if this biomass is in fact a perfect substrate for the creation of this type of renewable energy systems. However, seagrasses are biomasses that are more structurally and systematically correlated to terrestrial plants than marine algae (Orth, 2006).

The purpose of our study was to compare three different types of biomass (climbing vines, marine macro-algae and seagrasses) to determine which is a more efficient and effective biomass for creating a renewable energy system. Furthermore, we isolated and described the community of anaerobic alginate degraders that might be present in different natural terrestrial environments. The description of these microorganisms could help improve the system in which the marine algae are used as primary organic material for the generating the desired biofuel.

Literature Review

Technological advances and the creation of an industrialized society have developed a high dependency on resources like fossil fuels to supplement the energetic demands that result from these activities. Because fossil fuels like petroleum are finite resources; their high consumption has created a shortage of these types of products. As a result, there have been searches for energy sources that are renewable and have the capacity to substitute fossil fuels, and concomitantly offering the same or a better energetic rendition. Biofuels, a renewable energy source, have emerged as one of the most important sustainable fuel sources and are considered an essential way of progress for reducing greenhouse gas emissions, improving air quality and finding new energetic resources (Nigam, 2011). Even though they could be a good potential energy source, the actual development and production of these biofuels are too expensive in comparison with the developed industry of fossil fuels (Alvira, 2010). These biofuels are currently developed by the degradation of a variety of food products creating a competition for the destination of the crops harvested. To eliminate this problem it has been proposed the use of lignocellulolytic biomass to generate biofuels, like bioethanol (Kim, 2004).

Biofuels and bio-products produced from plant biomass would mitigate global warming because the release of CO₂ after burning equals the CO₂ absorbed by the plant during photosynthesis and thus the net production of CO₂ is close to zero (Naik, 2010). The selected biomass for production of such biofuels may be converted to a variety of energy forms including heat (via burning), electricity, hydrogen, ethanol, methanol, and methane (Chynoweth, 2001). One of the energy forms used for biofuel

generation, methane, could be a competitive alternative in both energy efficiency and environmental impact, utilizing lignocellulosic biomass as degradation (Pakarinen, 2008). Methane has a calorific value, which is the heat produced as a result of its combustion, of 53 MJ/kg while gasoline's is only 46 MJ/kg (Scott, 1999). This demonstrates that methane has the potential to be an efficient and better energy source, more than what is currently produced with fossil fuels as long as the concentration of this biogas is above 30% (Nikiema, 2005). Moreover, an advantage of the use of methane, which is not seen while using fossil fuels or even bioethanol, is that the product is purified by its own production (Chynoweth, 2001). The energy and economic input invested in bioethanol and gasoline industries could be avoided while using methane as a renewable energy system. The overall methane fermentation process is comprised of two principal steps: conversion of complex organics to volatile organic acids, and fermentation of these acids to methane and carbon dioxide (Foree, 1970). The primary focus for the creation of these types of biofuels is the use of biomass that does not interfere with products that have other industrial purposes, like the food industry. Therefore, lignocellulosic feedstock can offer the potential to provide novel biofuels, biofuels of the "second-generation" (Naik, 2010).

Vines can be defined as climbing plants that are deep-rooted in the soil and the stems alone are incapable of maintaining themselves rigid, needing support for growth (Acevedo-Rodríguez, 2005). Although many scientists have overlooked climbing plants, they are a promising group of plants to explore their biofuel value (Putz, 1991). Climbing vines can represent 20 to 40% of the diversity in a Tropical Forest, but in a Temperate Forest they can represent up to 7% of the diversity present (Acevedo-Rodríguez, 2002; Putz, 1991). In tropical regions the diversity of climbing

vines is high thus generation of this type of biomass is favored for industrial purposes. Vines can contribute to tree mortality, slow their growth, and cause distinctive injury to stems and branches of emerging trees (Granados, 2002). However, climbing vines could be the preferential biomass for the creation of second-generation biofuels. The combination of the dispersal, rapid growth, obtaining of resources and climbing capability, gives vines a competitive advantage over self-supporting plants (Paul, 2011). Climbing vines have vessels much larger than trees, for which all the water resources available will be obtained first by the climbing vines thus an advantage for growth (Putz, 2012). Though cellulosic feedstock does not use human food resources, they still require intensive care in terms of fertile land, watering, and other agricultural inputs for their production (Sander, 2010). However, climbing vines can grow on both low and rich nutrient soils and possess a variety of mechanisms that permit them to climb any surface, needing almost no care or input for their production (Putz, 1991; 2012). The use of climbing vines certainly has advantage in terms of production; however, the degradation of climbing vines requires specific mechanisms and enzymes as a result of their complex structure. For these reasons there have been searches for other biomasses that may require less effort for their degradation, like marine algae.

The chemical composition of marine algae includes complex sugars, like alginate, laminarin and mannitol, which are easily converted to intermediaries for methane production in anaerobic environments (Shekhar, 2012). In natural situations, algae enter dark-anaerobic environments where, due to conditions unfavorable for growth, they die and decompose (Foree, 1970). Bird et al. (1990) conducted a study determining the relationship of the composition of marine algae and their potential

methane production. The results indicated that species like *Sargassum* spp. had an actual relative low methane production and proved to be a poor biomass for methane production, in comparison to its theoretical methane production value. While *Sargassum* spp. could have a low methane production, it could be counter balanced by the fact that the marine algae can be generated in a short period of time and are excellent candidates for algae farm cultivation (Hanisak, 1987). Other researches, as Vergara-Fernández et al. (2008), determined that other species of marine macro-algae, like *Macrocystis* spp. and *Durvillea* spp., are a great organic material for methane production. They proposed to create a two-phase anaerobic reactor system, for a greater methane production. This theory is based on the fact that the methanogenic phase could be the limiting step of the anaerobic degradation of marine algae because of a low removal of chemical oxygen demand (Vergara-Fernández, 2008). Due to high growth rates of marine macro-algae they could be an excellent material as primary biomass for generation of methane. Marine macro-algae can generate up to 13.1 kg of dry weight in a period of 7 months in comparison to the growth rate of terrestrial plants (Kraan, 2010). Also, like in climbing vines, this biomass requires low quantity of nutrient and low energy input for its cultivation (Kraan, 2010); creating a system that could be a cost-effective industry competing efficiently against fossil fuel industries.

The biomass of marine algae is up to 30-40% composed of alginate or sodium alginate. Alginate is made of linear copolymers of 1,4- β -D-mannuronic acid (M) and α -L-guluronic acid (G) (Dettmar, 2011). The arrangements of the M and G blocks depend on the source from which it is obtained (Gómez, 2009) and its degradation is completed by an alginate lyase that acts specifically on the 4-O-linked glycosidic

linkage of alginate (Tang, 2008). Previous studies have determined that alginate in anaerobic conditions is converted to CO₂, acetate, ethanol, formate, succinate, butyrate, and methane (Moen, 1997). However, there is not enough literature describing the actual alginate degraders in anoxic environments. The understanding and description of these microorganisms could help develop a more efficient system for the degradation of marine algae.

Objectives

1. Compare tropical climbing vines, marine macro-algae and seagrasses as primary feedstock for the anaerobic microbial bioconversion to methane.
2. Describe the anaerobic microbial community present in the different microcosms enriched with various natural habitats using DNA based molecular tools.
3. Isolate and describe the anaerobic alginate degraders present in different natural habitats.

Materials and Methods

1.1 Experimental Inoculum

The experimental anaerobic microcosms were inoculated with a selection of microorganisms from three experimental samples of anaerobic community from: algae, alginate and cellulose degraders. The experimental inoculum was constructed by removing 10 mL of culture media from the enrichments previously described and combining them in a sterile and anaerobic serum bottle. The atmosphere in the serum bottle was exchanged with N₂ gas using strict anaerobic techniques (Balch and Wolfe, 1976).

1.2 Anaerobic microcosms

Marine algae and seagrass were collected from Balneario Caña Gorda in Guánica, Puerto Rico (GPS coordinates: 17.952256, -66.884897), from the shore (Figure 2) and later identified in the laboratory. Tropical climbing vines were collected in Mayagüez, Puerto Rico (GPS coordinates: 18.209968, -67.136347; 18.213826, -67.136229; 18.213001, -67.137951; 18.212139, -67.11003) along different roadsides and pastures (Figure 1, B-E) and later identified in the laboratory. Anaerobic microcosms were prepared with anaerobic medium as previously described by Jackson (1999). In an anaerobic chamber, 0.5 g, of previously dried, marine algae, seagrass and climbing vines and 28 mL of anaerobic media were placed in a serum bottle and sealed with a sterile butyl rubber stopper and an aluminum crimp. In a gassing station, using strict anaerobic techniques, the serum bottles were flushed with N₂:CO₂ gas (80:20 v/v) and their atmosphere was maintained to 10 psi (Balch, 1976).



Figure 2. Collection localities for marine algae, seagrass and tropical climbing vines. Map of Puerto Rico showing the towns were samples were obtained in (1) Mayagüez, Puerto Rico and (2) Guánica, Puerto Rico.

After mixing the experimental inoculum 2 mL were injected into each experimental replicate using standard sterile and anaerobic techniques (2/30mL= 6.7% inoculum). The source of biodegradable biomass consisted of three types of marine algae (*Sargassum fluitans*, an unidentified green marine algae, *Gracilaria* sp.) and two species of seagrasses (*Syringodium filiforme*, *Thalassia testudinum*). The species of marine algae analyzed are classified as Phaeophyta (brown algae) and Rhodophyta (red algae). The five genera of tropical climbing vines were *Desmodium incanum*, *Dioscorea bulbifera*, *Cayaponia racemosa*, *Epipremnum pinnatum*, and *Cissus verticillata*. This experimental set-up was repeated for every marine algae, seagrass and climbing vines analyzed separately. The experimental set-up was incubated at 30⁰C in the absence of light and without shaking.

1.3 Chemical Analysis

The methane production was measured every week as an indication of microbial activity towards the degradation of the biomass. Methane production was analyzed using a Gas Chromatograph (Shimadzu GC-2010) with a stainless steel column (Porapak N 80/100, 10ft x 1/8 in ss, detector FID) and it followed Bastviken's (2004) method. Every week, during the experimental period, volatile fatty acids production was measured using a High Performance Liquid Chromatography. To analyze the production, accumulation and/or degradation of organic volatile fatty acids, 0.5 mL of each sample was removed and placed in a sterilized Eppendorf tube and stored at -20°C. Before analysis, the sample was thawed at room temperature, and then 0.1 g of Dowex (Acros Organic; New Jersey, USA) was added to the sample and centrifuged at 13,200 rpm for 5 minutes to remove any impurities. The supernatant of the sample (40 µL) was injected in the equipment, which is composed of a Rezex ROA-Organic Acid H⁺ (18300 by 7.8 mm, 10 uL) column, a UV detector at 210 nm, and a 0.005 N sulfuric acid mobile phase following Jackson's (1999) method.

1.4 Molecular Analysis

A. DNA Extraction

The description of the anaerobic community present was based on a molecular analysis of the experimental set-ups of the marine algae, seagrasses and tropical climbing vines. The first step was to extract DNA from the samples, using the FastDNA Spin Kit for Soil (MP Biomedical; Ohio, USA) following the manufacturer

instructions. Since the cultures analyzed were fluid cultures, to obtain a pellet of cells 1.5 mL of fluid was extracted and centrifuged for 5 minutes at a constant velocity of 13,200 rpm. The supernatant was discarded and the previous step was repeated. After obtaining a pellet of cells only 400 uL were left on the microcentrifuge tube to continue the process as indicated on the manual.

B. Amplification of the Bacterial 16S rRNA gene

In our experimental samples, bacterial diversity was analyzed by PCR utilizing the GM5F-GC clamp (5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA0CGG-GGG-GCC-TAC-GGG-AGG-CAG-CAG-3') and DS907-Reverse (5'-ATT-ACC-GCG-GCT-GCT-G-3') (Muyzer, 1993). The thermo program for these set of primers consisted of a touchdown program in which the temperature decreased one degree from 63°C until it reached the desired temperature at 53°C. The program consisted of: 4 min at 94°C; 30 cycles of 1.25 min at 94°C, 45 sec at 53°C, and 2 min at 74°C, and a final extension step of 10 min at 72°C. The PCR Master Mix consisted of: 10 µL of 5X PCR buffer (Promega®), 5 µL of [2.5mM] dNTP's mix (New England Biolabs, Massachusetts), 5 µL of [25mM] of MgCl₂ (Promega®), 0.50 µL of each primer [2pmol/uL], 0.2 µL of FlexiTaq DNA polymerase (Promega®) [5U/µL], 2 µL of DNA template (with a concentration of 9.6 to 36.0 ng per µL) and sterile deionized distilled water to complete the reaction to a final volume of 50 µL. The end result was the amplification of a 550 base pair fragment of the 16S rRNA gene. The confirmation of expected size was resolved by electrophoresis using a 1.8% agarose gel, ran for 1.5 hours at 115v which were visualized and documented in a VersaDoc System (Bio-Rad®, USA). As positive control for our PCR, DNA from

grown cultures of *Syntrophus aciditrophicus* and *Desulfovibrio vulgaris* were extracted and amplified.

C. Amplification of the Archeal 16S rRNA gene

The molecular analysis of the methanogenic archaea present in our experimental samples was performed utilizing the 1106F (5'-TTW-AGT-CAG-GCA-ACG-AGC-3') and 1378R primers with GC Clamp (5'-TGT-GCA-AGG-AGC-AGG-GAC-3') and the thermo program previously described by Watanabe (2006). The PCR Master Mix for the Archeal 16S rRNA gene consisted of: 10 µL of 5X PCR Buffer (Promega®), 1.25 µL of [10mM] dNTP's mix (New England Biolabs, Massachusetts), 4 µL of [25mM] MgCl₂ (Promega®), 10 µL of each primer at [2pmol/µL], 0.4 of FlexiTaq DNA polymerase (Promega®) [5U/µL], 2 µL of DNA template (with a concentration of 9.6 to 36.0 ng per µL) and the reaction with a final volume of 50µL was completed with sterile deionized distilled water. The end result was the amplification of a 322 base pair fragment of the 16S rRNA gene. The confirmation of expected size was resolved by electrophoresis using a 1.8% agarose gel, ran for 1.5 hours at 115V, and visualized and documented in a VersaDoc System (Bio-Rad®, USA). As positive control for our PCR, DNA was extracted and amplified from a grown culture of *Methanospirillum hungatei*.

D. Denaturing gradient gel electrophoresis (DGGE)

After obtaining the amplification for both Bacterial 16S and Archaeal 16S, PCR products were then separated by DGGE, as previously described by Muyzer, using the D-code Universal Mutation Detection System (Bio-Rad®, USA), (1993). Profiles for the Bacterial and Archaeal 16S genes were obtained in denaturing gradient gels that

ranged from 20 to 60% of formamide and urea (respectively) with an 8% acrylamide solution. Both gels were run at 50V until the instrument reached 60°C, there after the voltage was augmented to 100V for 12 hours at 60°C. The gel was stained in a solution of Ethidium Bromide for 5 minutes, rested in 1X TAE for 15 minutes and visualized in a VersaDoc System (Bio-Rad, USA). Results obtained were compared with our positive controls that were: *S. aciditrophicus* and *D. vulgaris* for Bacterial 16S rRNA genes and *M. hungatei* for Archaeal 16S rRNA . DGGE profiles were then compared based on the presence or absence of the band selected in the lane. After constructing a presence/absence matrix, dendograms of neighbor-joining tree were analyzed with Paleontological Statistics Program (PAST) as previously described by Hammer (2001).

1.5 Isolation of anaerobic alginate degraders

The natural habitats selected for this study were sediments from: the Lucchetti Reservoir, a mechanical workshop, Guánica's Dry Forest, a Rice Paddy Field, and an oxidation pond from a Coffee Paddy Field; and a Rumen Fluid sample. Anaerobic medium containing 1% of alginate as sole source of carbon and energy were enriched with samples from the natural habitats previously mentioned. The preparation of the anaerobic 1% alginate medium was achieved by adding: 10 g of sodium alginate (SFAC; Missouri, USA) to the anaerobic medium previously described. After adding the sodium alginate, pH was adjusted to 7.2-7.5 (using a 1M solution of HCl if it was too basic or 1M of NaOH if it was too acidic), and served in anaerobic serum bottles or anaerobic tubes. Every week methane production in the samples was monitored

and measured using a GC method described in the previous section (1.3). After methane production stabilized, the samples were purified by serial dilutions in 1% anaerobic alginate medium as previously described in this section. After two consecutive serial dilutions, DNA was extracted using the FastDNA Spin Kit Soil (MP Biomedical; New Jersey, USA). Molecular analysis of the Bacterial and Archaeal 16S genes and DGGE analysis were performed in a similar manner previously described. To isolate discrete colonies of the microorganisms that were present in our alginate samples, roll tubes were performed (Balch and Wolfe, 1976). The roll tubes were created by using an anaerobic medium previously described (Jackson, 1999), and by adding 2% of agar to solidify the medium. After sterilizing the medium, 0.5 mL of the samples were transferred to 9.5 mL of the roll tube. The tubes were placed horizontally in a tube roller (custom built) until the medium had solidified (usually under a minute). The roll tubes were incubated at 30°C in the absence of light until individual colonies were visible. After growth, isolated colonies were transferred to the anaerobic 1% alginate medium previously described in an anaerobic chamber. After inoculation, in a gassing station using anaerobic techniques, the atmosphere of the serum bottles were maintained to 20 psi of N₂:CO₂ (80:20 v/v) (Balch and Wolfe, 1976). The pure cultures were incubated at 30°C in the absence of light and methane production was monitored weekly. The morphological description of the microorganisms was performed by Phase of Contrast Microscopy (Leica DMI3000B Microscope) at a 100 X magnification and photographed by the Micrometric SE Premium Program.

Results

2.1 Methane production

Methane production for the anaerobic microcosms with tropical climbing vines, marine algae and seagrass were monitored for 108 days. In terms of comparing the aquatic biomass, which were composed by microcosms containing marine algae or seagrasses, the species with the highest methane production was *Syringodium filiforme* with 21% of methane produced (Figure 3). Based on LSD Fisher Statistical Test there was a significant difference between two species of the aquatic biomass, *Syringodium filiforme* and *Sargassum fluitans* (Table 1). These microcosms represent the highest and the lowest production of methane, with a difference of 6.4% of methane. However there is no significant difference between *Thalassia testudinum*, *Syringodium filiforme*, *Gracilaria* sp. and the unidentified green marine algae (Table 1). Based on methane production, the highest energetic rendition came from microcosms with *Syringodium filiforme* where 3.60 W/h were produced from the degradation of 0.016 g/mL of the biomass (Table 2).

Contrary to microcosms with aquatic biomass, LSD Fischer Statistical test established that there was no significant differences of methane production among four of the species of tropical climbing vines analyzed (Table 3). The degradation of *Cissus verticillata* had the lowest rendition and a significant difference from those of *Dioscorea bulbifera* and *Desmodium incanum*, but not from the degradation of *Epipremnum pinnatum* and *Cayaponia racemosa* (Table 3). Taking out *Cissus verticillata* microcosms, methane production in the other microcosms ranged between 44 to 50% with a mean production of 47.3% of methane (Figure 4). The highest rendition was obtained from *Dioscorea bulbifera*, since from every 0.016 g/mL of

biomass degraded 50% of methane was produced and 8.37 W/h of energy could be obtained (Table 4). Nevertheless when comparing aquatic and terrestrial biomasses, it is clear that there is a significant difference in methane rendition between these two sources (Table 5). In terms of methane production, the highest production was again obtained from the vine *Dioscorea bulbifera*, which produced three times more methane than the marine algae (Figure 5).

There was also a difference in terms of the adaptation period for the microbial communities present in the three types of biomasses analyzed: marine algae, seagrasses and tropical climbing vines. In the marine algae microcosms, methane production commenced after 22 days, while in the seagrasses microcosms the production of methane started only after 8 days (Figure 3). In these previous microcosms methane production reached the highest after 67 days of degradation, while after this period methane production was constant (stable). However in microcosms containing tropical climbing vines the adaptation period could be divided into three different phases: 8, 22 and 50 days (Figure 4). The first period, which was after 8 days, only one microcosm exhibited this pattern, *Desmodium incanum*. The second adaptation period was after 22 days observed in microcosms with *Dioscorea bulbifera* and *Epipremnum pinnatum*. The remaining two species of climbing vines, *Cayaponia racemosa* and *Cissus verticillata*, presented an adaptation period of 50 days. In the same way that the adaptation period could be divided in three categories, methane production stabilization could be divided correspondingly. For *Desmodium incanum* microcosms, in which the microbial community needed only 8 days to adapt, methane production was stabilized after 67 days, similar to the behavior observed in seagrass microcosms. However, the microcosms containing *Dioscorea bulbifera* and

Epipremnum pinnatum, reached stability after 87 days, while both *Cayaponia racemosa* and *Cissus verticilata* took more than a 108 days to stabilize its methane production.

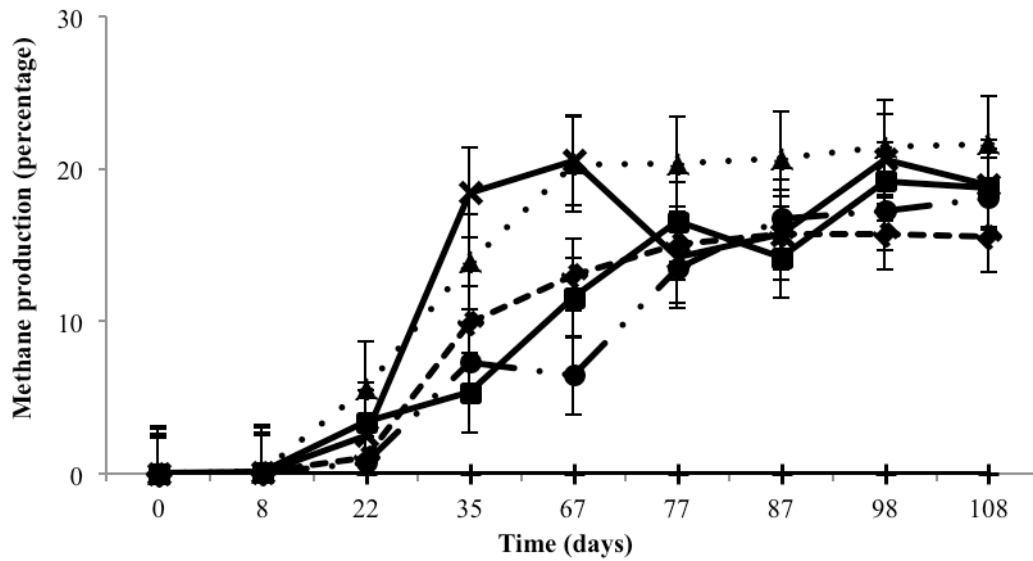


Figure 3. Methane production based on the anaerobic degradation of different species of marine algae and seagrass as primary biomass. Standard error demonstrates that there is no significant difference in the use of *Gracilaria* sp. (x), Unidentified marine algae (●), and *Thalassia testudinum* (■) as primary biomass for an anaerobic degradation except for the two species of *Sargassum fluitans* (◆) and *Syringodium filiforme* (▲).

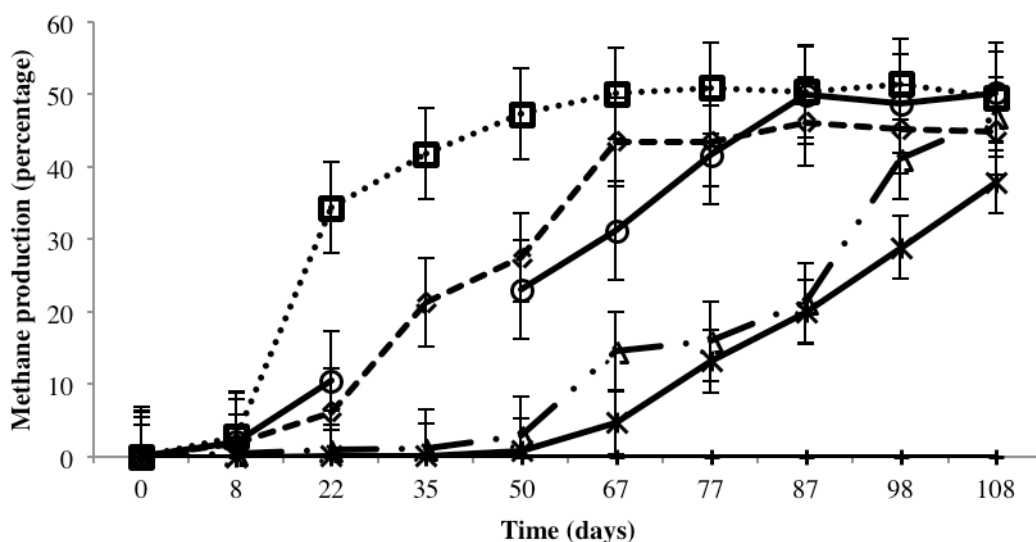


Figure 4. Methane production based on the anaerobic degradation of different species of tropical climbing vines as primary biomass. Standard error demonstrates that after 108 days there is significant difference between three species of climbing vines analyzed: *Desmodium incanum* (···□···), *Dioscorea bulbifera* (○) and *Cissus verticilata* (*); while there is no significant difference among the other climbing vines analyzed: *Cayaponia racemosa* (- · -△- · -) and *Epipremnum pinnatum* (---◇---).

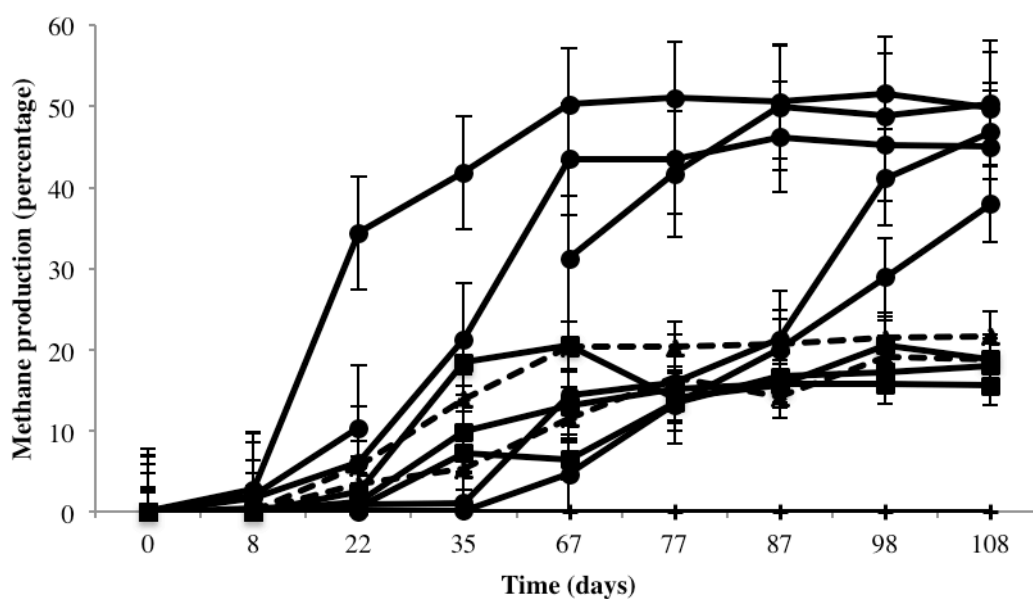


Figure 5. Comparison of the methane produce as the result of the anaerobic degradation of different species of tropical climbing vines, marine algae and seagrasses. Standard error demonstrates that there is significant difference between the methane produce from the climbing vines (●) and the marine algae (■) and seagrasses (---▲---). The tropical climbing vines are a more biodegradable biomass than the marine algae and seagrasses.

Table 1. LSD Fisher Statistical Test of the anaerobic microcosms degrading different species of marine algae and seagrasses. The statistical test demonstrates that there is a significant difference between two of the species present.

Test: LSD Fisher	Alfa=0.05	DMS=4.95806			
Error: 7.4273	gl: 10				
Biomass	Means	n	E.E		
<i>Sargassum fluitans</i>	15.60	3	1.57	A	
Unidentified marine algae	18.13	3	1.57	A	B
<i>Thalassia testudinum</i>	18.74	3	1.57	A	B
<i>Gracilaria</i> sp.	18.91	3	1.57	A	B
<i>Syringodium filiforme</i>	21.63	3	1.57		B
Means grouped with the same letter aren't significantly different (p > 0.05)					

Means grouped with the same letter aren't significantly different (p > 0.05)

Table 2. Energetic rendition based on the methane production of the anaerobic digesters enriched with different species of aquatic biomass (marine algae or seagrasses).

	Marine algae			Seagrass	
	<i>Sargassum fluitans</i>	Unidentified marine algae	<i>Gracilaria</i> sp.	<i>Syringodium filiforme</i>	<i>Thalassia testudinum</i>
Methane produced (grams)	0.1559	0.1812	0.1890	0.2163	0.1873
Energy production (W/h)	2.60	3.02	3.15	3.60	3.12
Biomass required (kg per month)	147.07	127.15	121.90	106.67	123.07

*Methane production was converted to grams based on the notion that the total of the gas volume available in the headspace of the microcosms is equal to 100%, thus 100% would be equal to 1g. The biomass required per month is based on the energetic consumption of a household consuming 240kW/h in a month.

Table 3. LSD Fisher Statistical Test of the anaerobic microcosms degrading different species of tropical climbing vines. The statistical test demonstrates that there is no significant difference between four of the species analyzed, while there is significant difference between *Cissus verticilata* and *Desmodium incanum* and *Dioscorea bulbifera*.

Test: LSD Fisher Alfa=0.05 DMS=1.87091					
Error: 1.0576		gl: 10			
Biomass	Means	n	E.E		
<i>Cissus verticilata</i>	37.96	3	3.26	A	
<i>Epipremnum pinnatum</i>	44.94	3	3.26	A	B
<i>Cayaponia racemosa</i>	46.83	3	3.26	A	B
<i>Desmodium incanum</i>	49.63	3	3.26		B
<i>Dioscorea bulbifera</i>	50.23	3	3.26		B
Means grouped with the same letter aren't significantly different (p > 0.05)					

Table 4. Energetic rendition based on the methane production of the anaerobic digesters enriched with different terrestrial biomasses (tropical climbing vines).

	<i>Desmodium incanum</i>	<i>Dioscorea bulbifera</i>	<i>Cayaponia racemosa</i>	<i>Cissus verticilata</i>	<i>Epipremnum pinnatum</i>
Methane produced (grams)	0.4962	0.5023	0.4683	0.3796	0.4493
Energy production (W/h)	8.27	8.37	7.80	6.32	7.48
Biomass required (kg per month)	46.43	45.87	49.23	60.75	51.33

*Methane production was converted to grams based on the notion that the total of the gas volume available in the headspace of the microcosms is equal to 100%. The biomass required per month is based on the energetic consumption of a household consuming 240kW/h in a month.

Table 5. LSD Fisher Statistical Test comparing anaerobic microcosms degrading marine biomass and tropical climbing vines. The statistical test demonstrates that there are significant differences between the marine biomass and the tropical climbing vines biomass.

Test: LSD Fisher Alfa=0.05 DMS=1.29480

Error: 0.5779

gl: 20

Biomass	Means	n	E.E		
<i>Sargassum spp.</i>	3.94	3	0.44	A	
UMA	4.08		0.44	A	B
<i>Thalassia spp.</i>	4.25	3	0.44	A	B
<i>Gracilaria spp.</i>	4.33	3	0.44	A	B
<i>Syringodium spp.</i>	4.33	3	0.44		B
<i>Cissus spp.</i>	4.64	3	0.44		C
<i>Epipremnum spp.</i>	6.79	3	0.44		C D
<i>Cayaponia spp.</i>	6.84	3	0.44		D
<i>Desmodium spp.</i>	7.04	3	0.44		D
<i>Dioscorea spp.</i>	7.0	3	0.44		D

Means grouped with the same letter aren't significantly different (p > 0.05)

2.2 Chemical Analysis

The accumulation of volatile organic acids appeared to be limited throughout the degradation of both marine algae and seagrasses. Microcosms containing seagrasses both presented the accumulation of acetate, whereas the other organic acids analyzed remained to a concentration lower than 1 mM (Figure 6, 7). In contrast in the marine algae microcosms only *Gracilaria* sp. presented the highest accumulation of acetate throughout the experimental period; it reached almost 20 mM of the organic acid (Figure 8, 9, 10). In both *Gracilaria* sp. (marine algae) and *Syringodium filiforme* (seagrass) the organic acid appears to be accumulated in the first 22 days of the degradation; being degraded later on to a concentration lower than 1mM (Figure 7, 8). Only in the microcosm containing biomass from *Gracilaria* sp. the degradation of acetate appears to be proportional to methane production (Figure 8). Moreover the accumulation and degradation of propionate seemed to oscillate throughout time. In the microcosms with *Thalassia testudinum* the degradation of acetate took the longest of all the microcosms analyzed, having been accumulated for 45 days (Figure 6).

Contrary to the marine algae and seagrasses microcosms, in microcosms containing tropical climbing vines there was a high accumulation of organic acids and in some microcosms this accumulation remained constant. Microcosms that degraded *Desmodium incanum*, accumulated acetate in the first 8 days; which its degradation was proportional to the methane production in this many days (Figure 11). However, the accumulation and degradation of lactate appeared to oscillate in the final phase of degradation (last 73 days) while butyrate and propionate stayed in lower

concentrations (Figure 11). In *Dioscorea bulbifera* microcosms, there was an extremely high concentration of lactate present from the beginning, which was degraded in a total of 8 days (Figure 12). However, acetate accumulated for a period of 22 days and was degraded slowly throughout 65 days, while the rest of the organic acids remained at minimal concentrations (Figure 12). In the microcosms that degraded *Cayaponia racemosa* biomass, acetate accumulated to concentrations as high as 60mM during the first 35 days of degradation, while after that period its degradation oscillated throughout the experimental period (Figure 13). Organic acids like butyrate and propionate were accumulated and degraded during the analysis, however the concentrations they reached were considerably lower in comparison to acetate (Figure 13). In *Cissus verticillata* microcosms, the accumulation of acetate was observed during the first 67 days reaching levels as high as 43 mM (Figure 14). However in the following 20 days it was partially degraded and remained at a concentration of 25mM. Acetate was subsequently degraded at the same time that methane was produced, although not proportionally. In the same matter as *Dioscorea bulbifera*, microcosms that contained *Epipremnum pinnatum* biomass, accumulated acetate (48mM) during the first 22 days, which thereafter it was not proportional to the methane production while the rest of the acids were maintained at low levels (Figure 15). However in the last 31 days, acetate was accumulated but to a much lower concentration than previously (Figure 15).

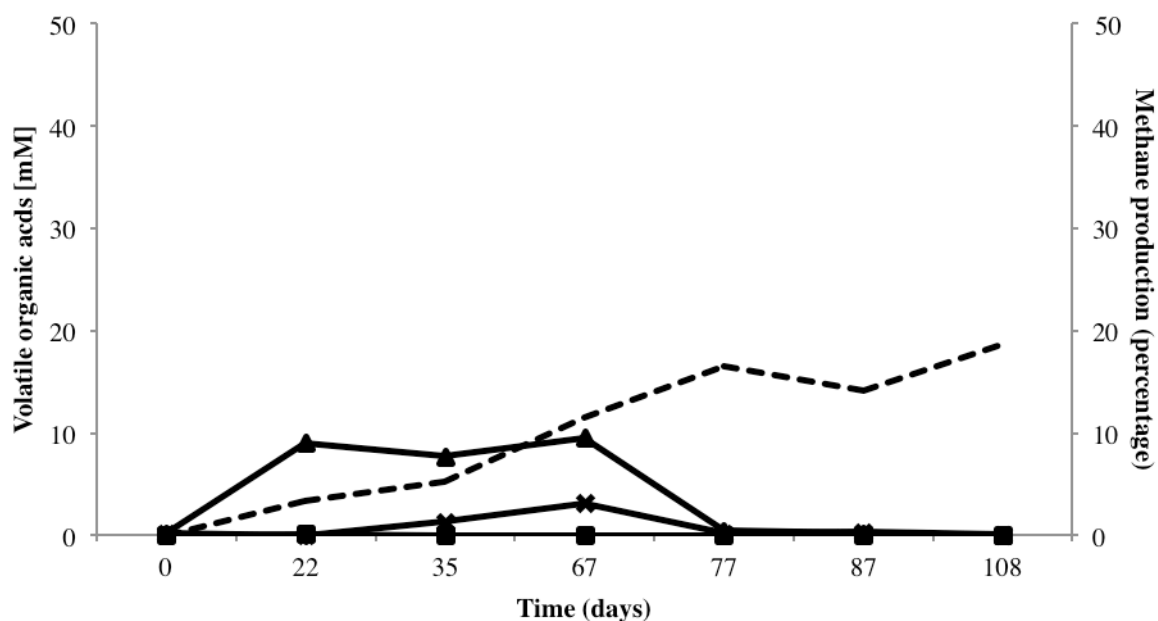


Figure 6. Chemical analysis by a High Performance Liquid Chromatography of the anaerobic degradation of the seagrass *Thalassia testudinum*. The graph demonstrates that acetate (▲) was accumulated for approximately 47 days, and afterwards was degraded to low levels. Lactate (◆), butyrate (■), propionate (×) and methane (---).

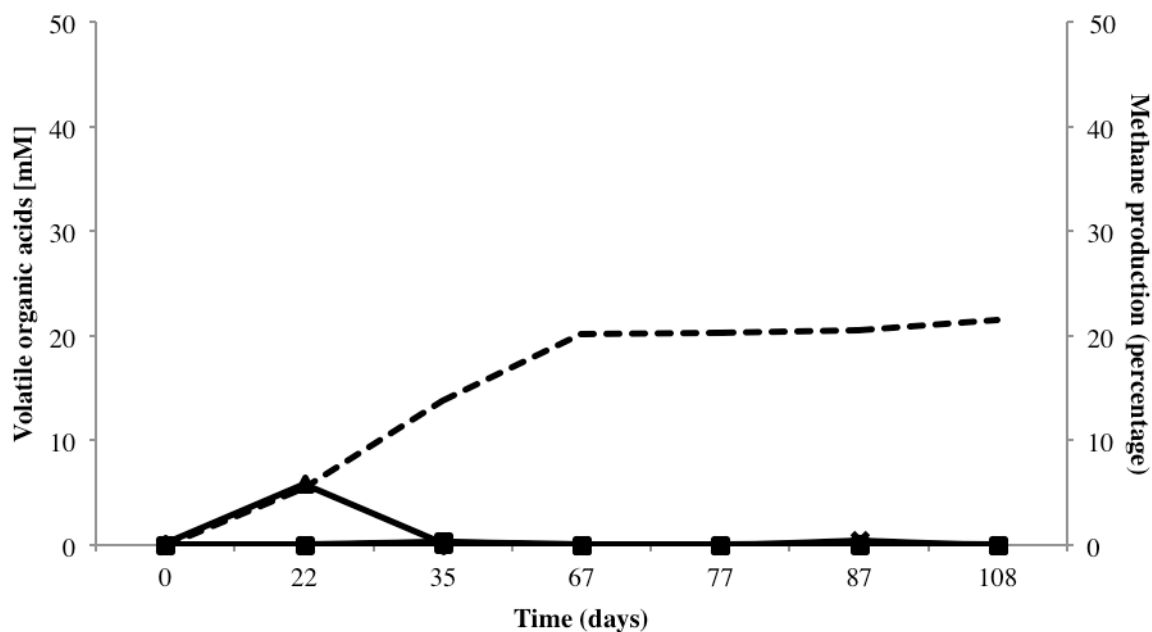


Figure 7. Chemical analysis by a High Performance Liquid Chromatography of the anaerobic degradation of the seagrass *Syringodium filiforme*. The graph demonstrates that there was no significant accumulation of volatile fatty acids in the microcosms. Lactate (◆), acetate (▲), butyrate (■), propionate (×) and methane (---).

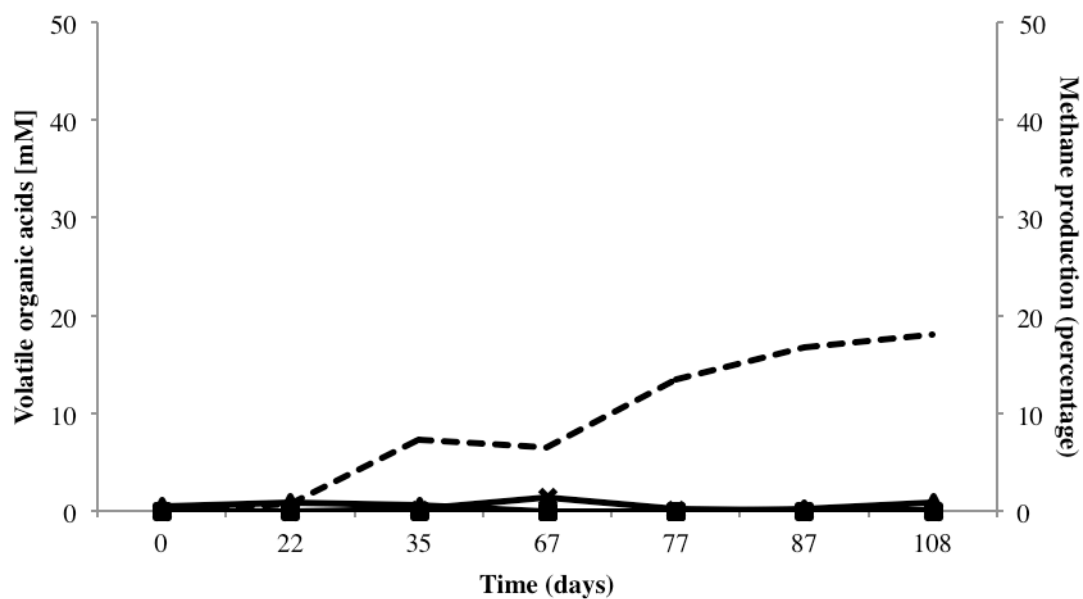


Figure 8. Chemical analysis by a High Performance Liquid Chromatography of the anaerobic degradation of the marine algae *Gracilaria* sp. The graph demonstrates that there was no accumulation of volatile fatty acids in the microcosms, except for acetate (▲) and propionate (×). Lactate (◆), butyrate (■), and methane (---).

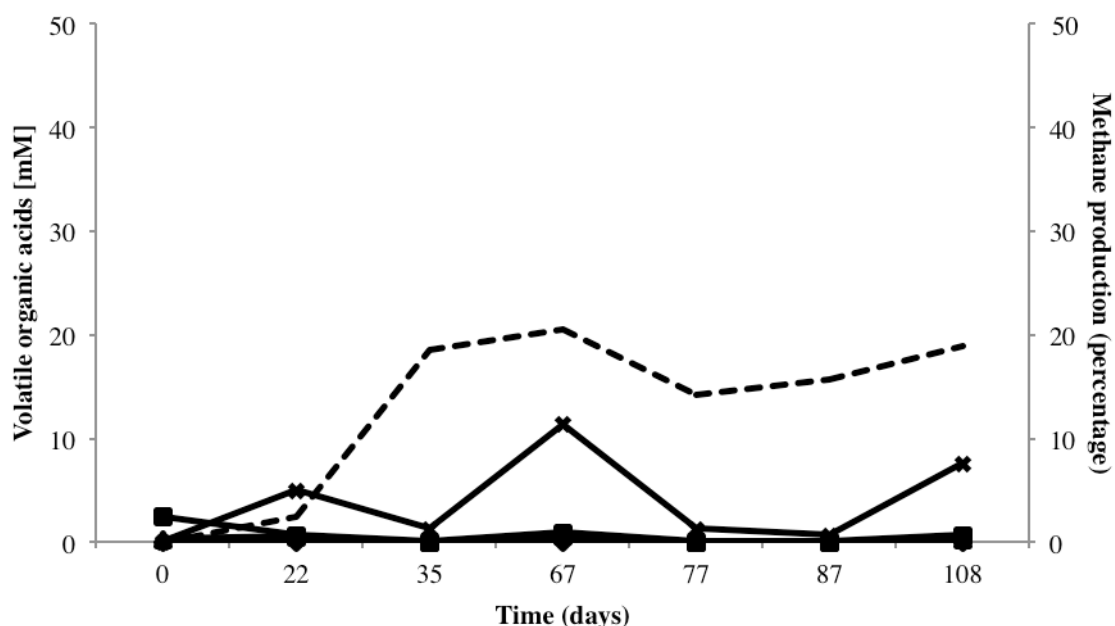


Figure 9. Chemical analysis by a High Performance Liquid Chromatography of the anaerobic degradation of an unidentified marine alga. The graph demonstrates that there was an intermittent accumulation and degradation of propionate (×) throughout time, while other acids were never accumulated. Lactate (◆), acetate (▲), butyrate (■), and methane (---).

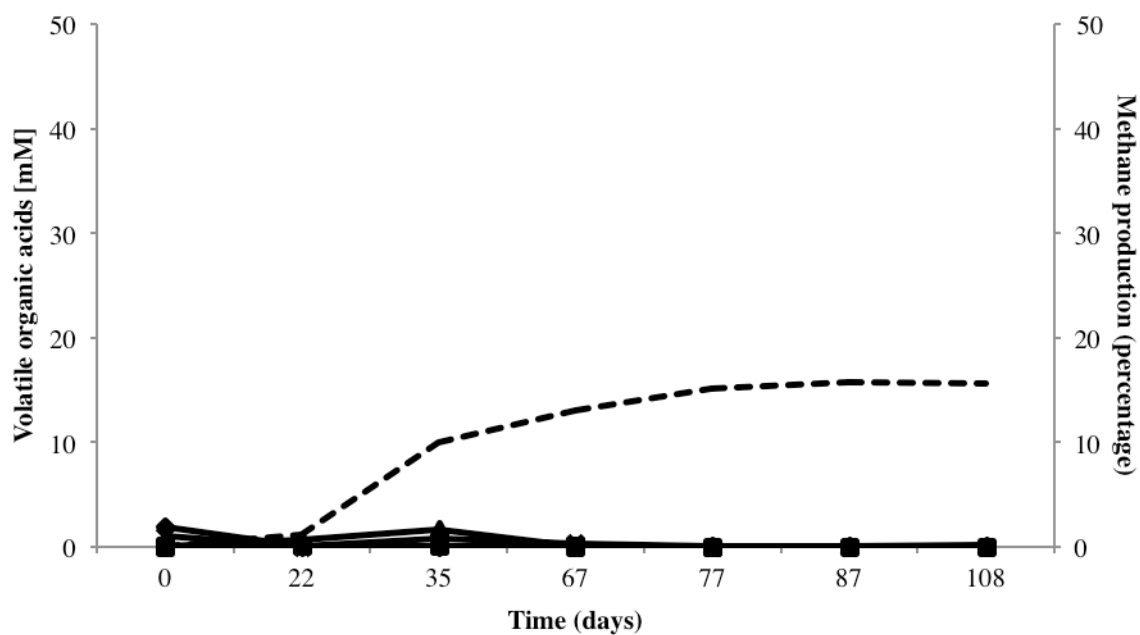


Figure 10. Chemical analysis of the anaerobic degradation of the marine algae *Sargassum fluitans*. The graph demonstrates that there was no accumulation of volatile fatty acids in the microcosm. Lactate (◆), acetate (▲), butyrate (■), propionate (×) and methane (---).

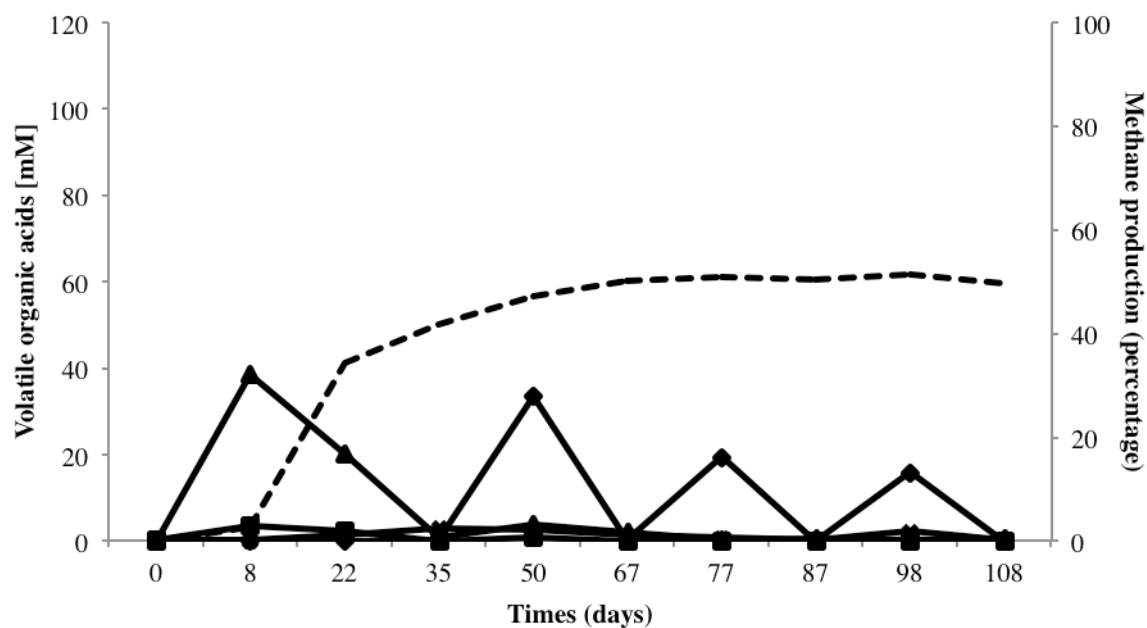


Figure 11. Chemical analysis of the anaerobic degradation of the tropical climbing vine *Desmodium incanum*. The graph demonstrates that there was a significant accumulation of volatile fatty acids in the microcosm, specifically acetate (▲) and lactate (◆). There was an intermittent production and degradation of lactate in the last 73 days. Butyrate (■), propionate (×) and methane (---).

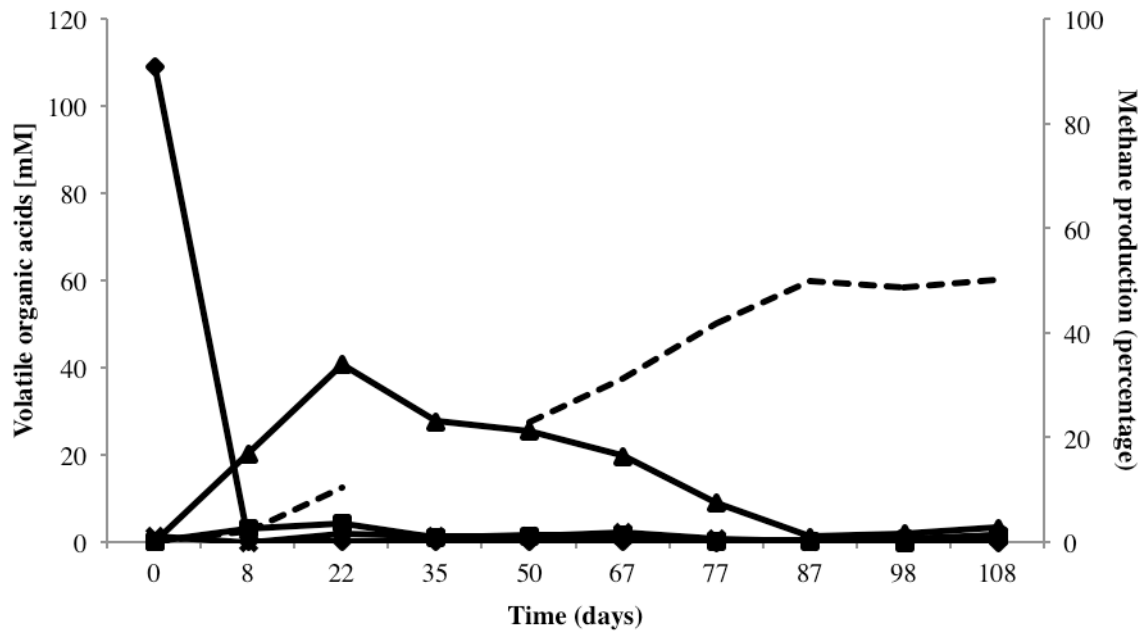


Figure 12. Chemical analysis of the anaerobic degradation of the tropical climbing vine *Dioscorea bulbifera*. The graph demonstrates that there was a significant accumulation of acetate (▲) in the first 22 days, while there was an aggressive degradation of lactate (◆) in the first 8 days. Butyrate (■), propionate (×) and methane (---).

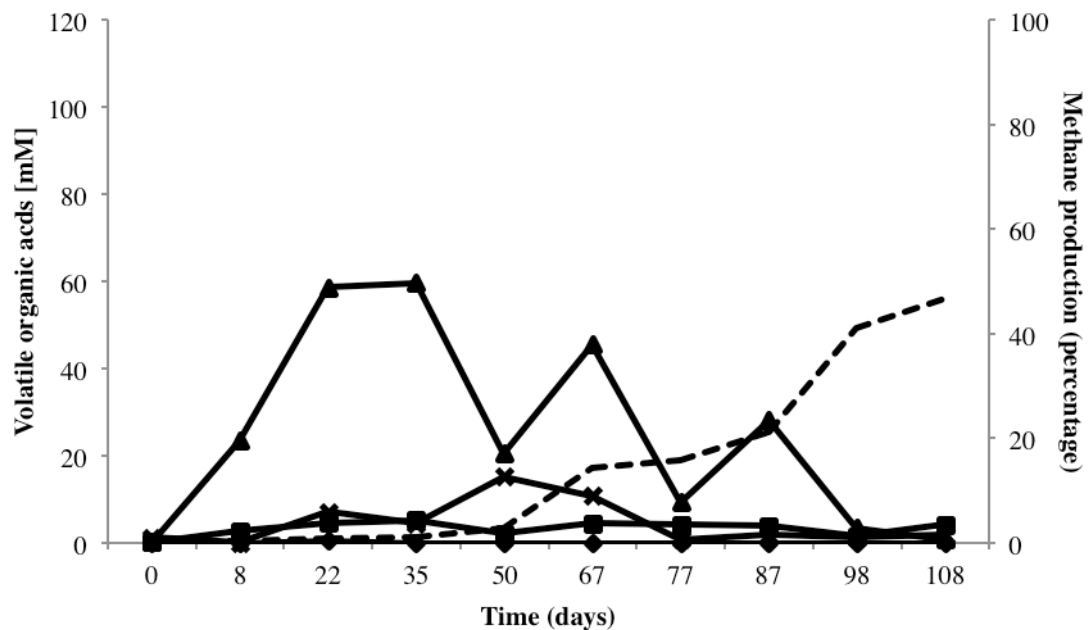


Figure 13. Chemical analysis of the anaerobic degradation of the tropical climbing vine *Cayaponia racemosa*. The graph demonstrates that there was a high accumulation of acetate (▲) in the first 35 days, where after this time there was an intermittent accumulation and degradation. Lactate (◆), butyrate (■), propionate (×) and methane (---).

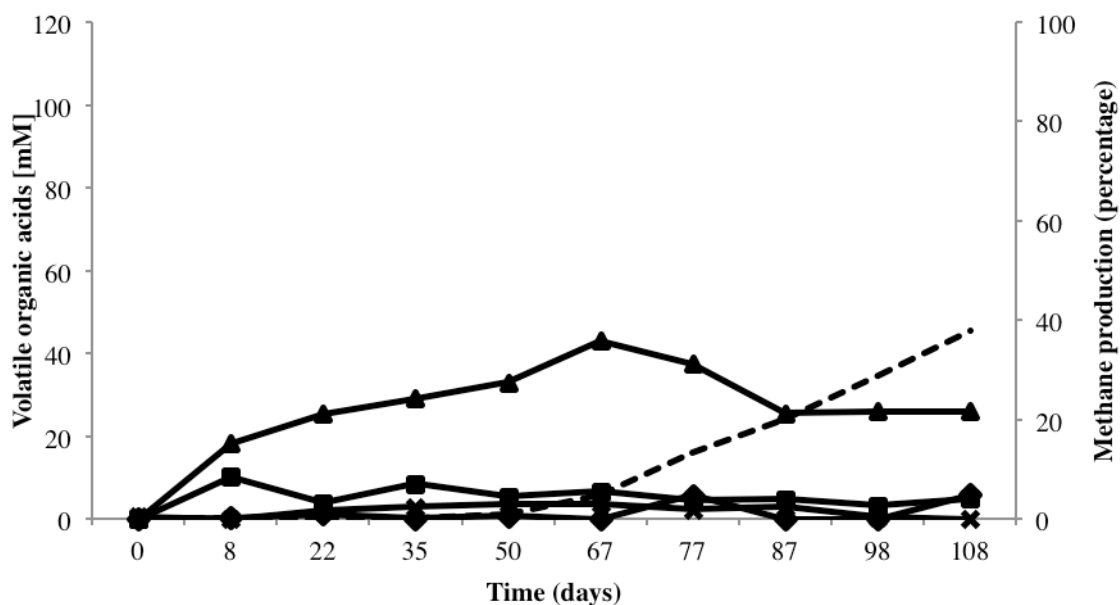


Figure 14. Chemical analysis of the anaerobic degradation of the tropical climbing vine *Cissus verticilata*. The graph demonstrates that there was a significant accumulation of volatile organic acids in the microcosm. Lactate (◆), acetate (▲), butyrate (■), propionate (×) and methane (---).

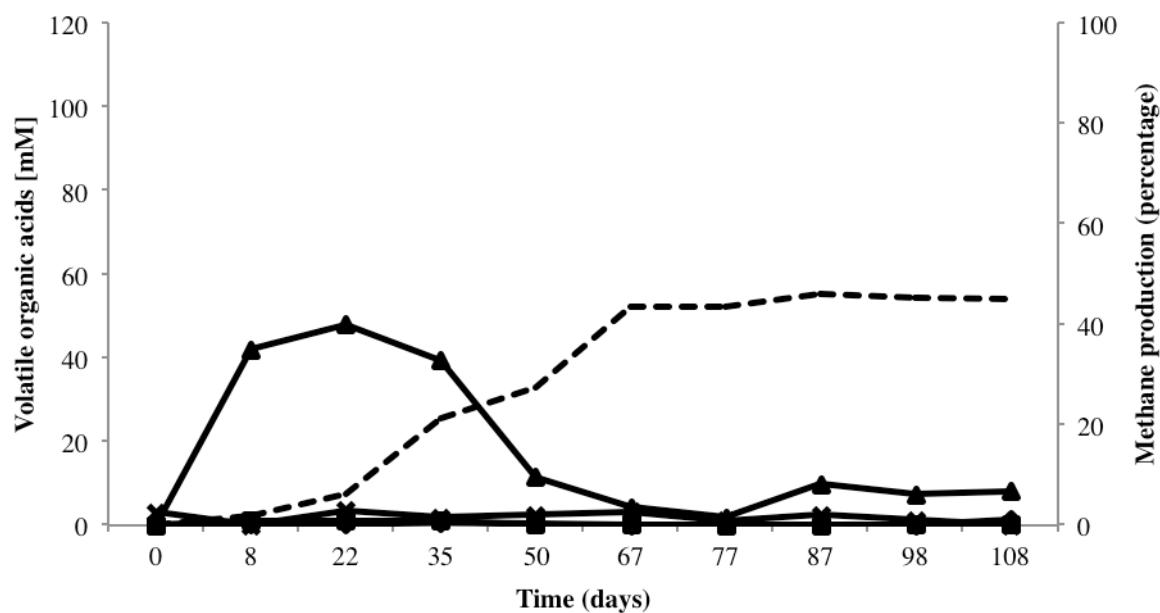


Figure 15. Chemical analysis of the anaerobic degradation of the tropical climbing vine *Epipremnum pinnatum*. The graph demonstrates that there was a significant accumulation of volatile organic acids in the microcosm. Lactate (◆), acetate (▲), butyrate (■), propionate (×) and methane (---).

2.3 Molecular Analysis

The experimental samples analyzed for the Bacterial 16S gene, using GM5F and DS907R primers, displayed the correct amplification product for both marine algae and tropical climbing vines microcosms (Figure 16). However, when the samples were analyzed for the Archeal 16S gene using the 1106F and 1378R primers, not all samples showed amplification (Figure 17). Samples that amplified with the previous primers from the marine algae microcosms were the experimental microcosms from: *Syringodium filiforme*, *Thalassia testudinum*, *Sargassum fluitans*, while all the experimental microcosms from the tropical climbing vines showed amplification.

DGGE analysis demonstrated that the bacterial communities present in the anaerobic microcosms degrading marine biomasses exhibited low diversity and homogeneity (Figure 18). These communities appeared to be dominated by two or three bacteria, specifically what appeared to be *Desulfovibrio vulgaris*. The sample with the highest diversity was the biomass of the unidentified green marine algae, which was not inoculated, a diversity that was not reflected on the experimental microcosm of that marine algae inoculated (Figure 18). Cluster analyses using Jaccard's similarity was performed to determine if the experimental microcosms exhibited the same patterns and were grouped within the same cluster or if they presented different patterns and were grouped into different clusters. The dendrogram demonstrated that in almost all microcosms both experimental and the unamended marine biomass were grouped within the same clusters, showing no differentiation between experimental microcosms and unamended microcosms (Figure 19). The only microcosm that both their unamended and experimental sample was not grouped in

the same cluster was the unidentified green marine algae since the diversity in the unamended was higher than in the experimental.

The archaeal community present in the experimental microcosms analyzed with the methanogen specific 16S gene primers demonstrated that the diversity was low as seen also with the bacterial community. However, the dominance in the experimental microcosms depended on the marine biomass that was being degraded (Figure 20). In the *Thalassia testudinum* microcosms, though the community present in the experimental microcosms was similar to the one in the unamended microcosms; the dominant Archaea came from the unamended microcosms. In contrast to the archaeal community present in the marine algae *Sargassum fluitans* though they are similar in both microcosms, the Archaea that dominated derived from the experimental inoculum (Figure 20). Cluster analysis with Jaccard similarity showed that in fact both experimental and unamended microcosms were grouped within the same cluster (Figure 21).

In terms of the bacterial community present, in the climbing vines experimental microcosms there was a higher diversity in comparison to the community present in the marine biomass microcosms (Figure 22). It appeared that the bacterium that dominated in the experimental microcosms derived from the experimental inoculum, and more importantly no matter what genera of climbing vines were analyzed the bacterium would have been the same. In this case, as observed in the marine biomass, the dominant bacterium is *D. vulgaris*. Cluster analysis using Jaccard's similarity revealed that there is no distinction between the community present in the marine biomass and the tropical climbing vines microcosms (Figure 23). Moreover, the

community present in the microcosm degrading *Epipremnum pinnatum* was identical to the one present in the *Syringodium filiforme* microcosm.

On the other hand, in terms of the methanogenic community present, DGGE demonstrated that there was a low diversity of microorganisms (Figure 24). In terms of dominance, the Archaea differed based on the type of climbing vine that was present in the microcosms, as was also seen in the marine biomass microcosms. However, the Archaea that appeared to dominate did not originate from the experimental microcosms but possibly from the biomass before they were inoculated (Figure 24). Nonetheless, when comparing the community present in both the inoculum and the experimental microcosms containing marine biomass, there was a higher diversity of Archaea in the climbing vines microcosms. Cluster analysis demonstrated that three microcosms contained similar communities and were grouped within the same cluster (*Dioscorea bulbifera*, *Epipremnum pinnatum* and *Cissus verticillata*). At the same time, the remaining two microcosms (*Cayaponia racemosa* and *Desmodium incanum*) were more similar to each other and to the microorganisms present in the experimental inoculum (Figure 25). When the archaeal community present in both biomasses is compared, it can be observed that the Cluster analysis grouped them together (Figure 26). The similarity of the community is based on the fact that the few dominant microorganisms present in the microcosms were derived from the experimental inoculum.

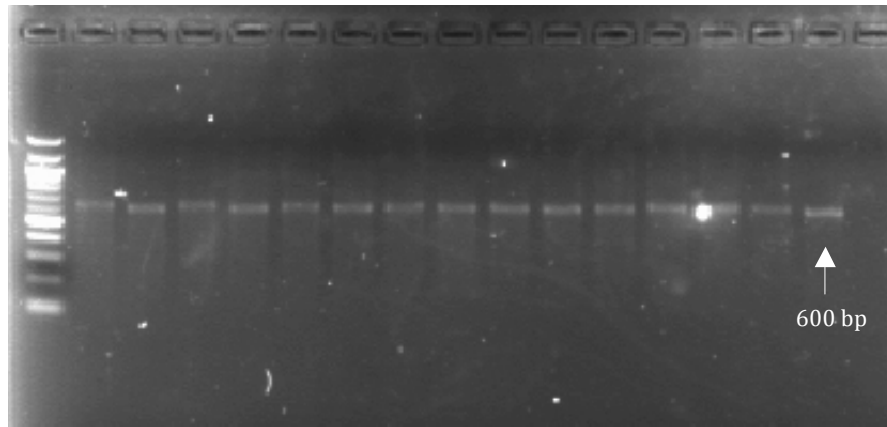


Figure 16. Agarose gel electrophoresis demonstrating a representative amplification of the Bacterial 16S rDNA gene of the samples analyzed (10 samples). The gel was a 1.8% agarose gel, ran for 1.5 hours at 115V.

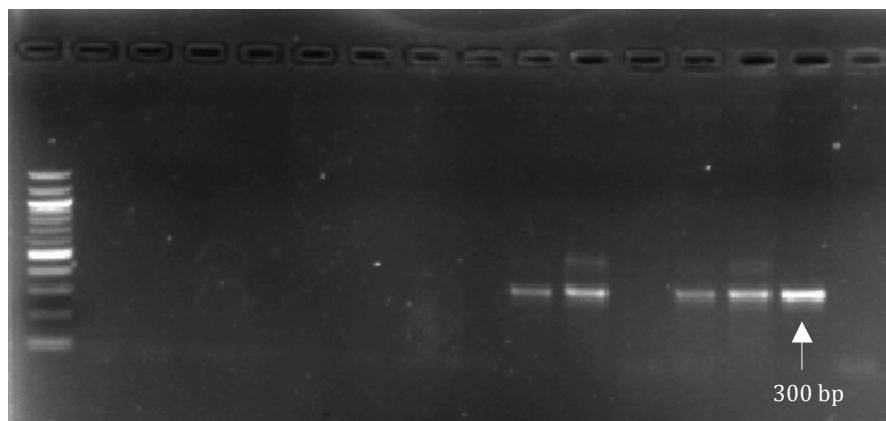


Figure 17. Agarose gel electrophoresis showing a representative amplification of the Archaeal 16S rDNA gene of the samples analyzed (10 samples). The gel was a 1.8% agarose gel, ran for 1.5 hours at 115V.

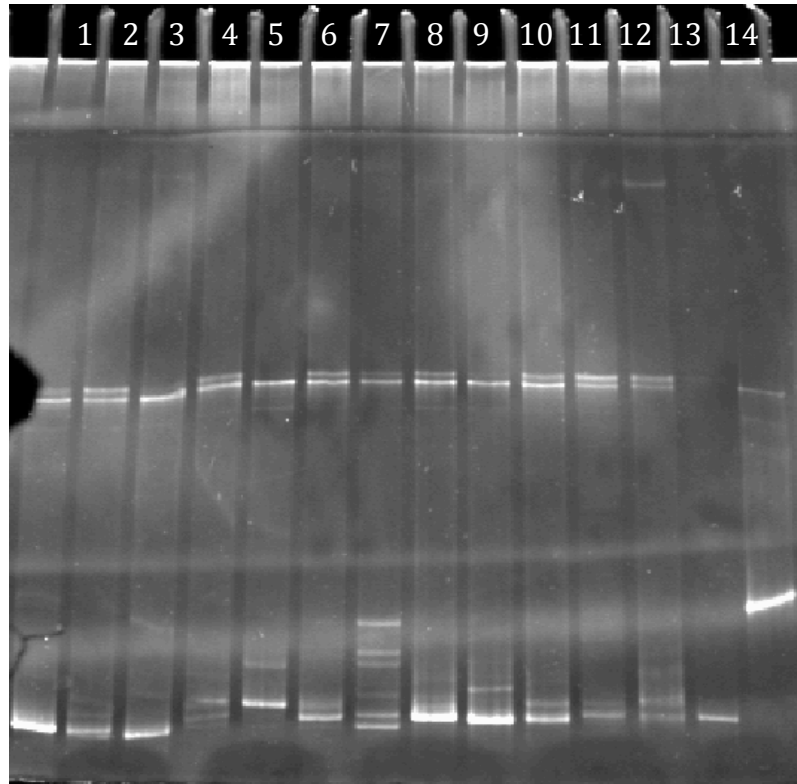


Figure 18. Denaturing gradient gel electrophoresis of the bacterial community present in the marine algae and seagrass anaerobic microcosms. The bacterial community of the microcosms presented a low diversity dominated mainly by two or three bacteria. The gradient of the gel presented was of 30-55% of bis-acrylamide at 60°C for 12 hours. Lane 1. *Syringodium filiforme* unamended, Lane 2. *Thalassia testudinum* experimental, Lane 3. *Thalassia testudinum* unamended, Lane 4. *Gracilaria* sp. experimental, Lane 5. *Gracilaria* sp. unamended, Lane 6. Unidentified marine algae experimental, Lane 7. Unidentified marine algae unamended, Lane 8. *Sargassum fluitans* experimental, Lane 9. *Sargassum fluitans* unamended, Lane 10. Mixture of the marine algae and seagrass biomasses, Lane 11. *Thalassia testudinum* experimental, Lane 12. Experimental inoculum, Lane 13. *D. vulgaris*, Lane 14. *S. aciditrophicus*.

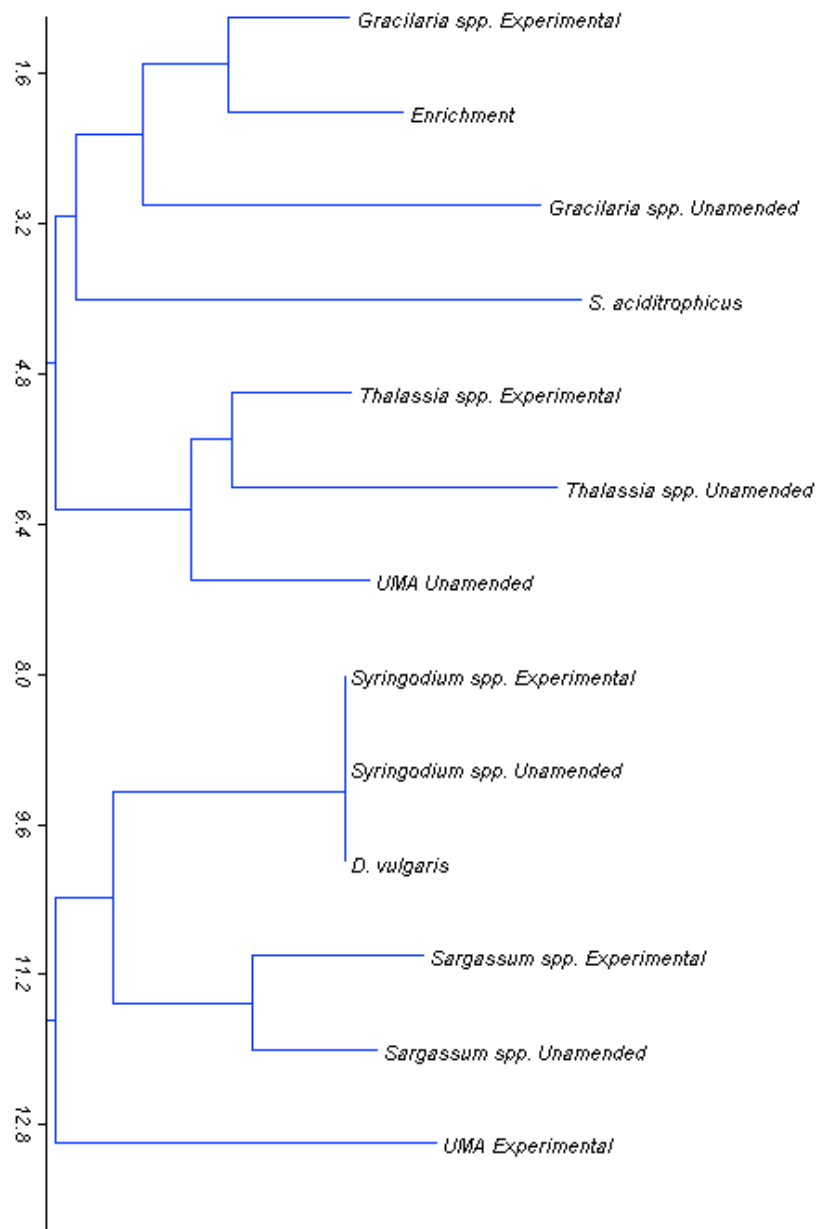


Figure 19. Cluster analysis with Jaccard's similarity of the bacterial community present in the marine algae and seagrasses anaerobic microcosms. The dendrogram demonstrated that the bacteria present in both the experimental and unamended microcosms are similar for which they are grouped together. In some genera of marine algae or seagrasses both experimental and unamended, were in the same cluster in some cases they were grouped in different clusters.

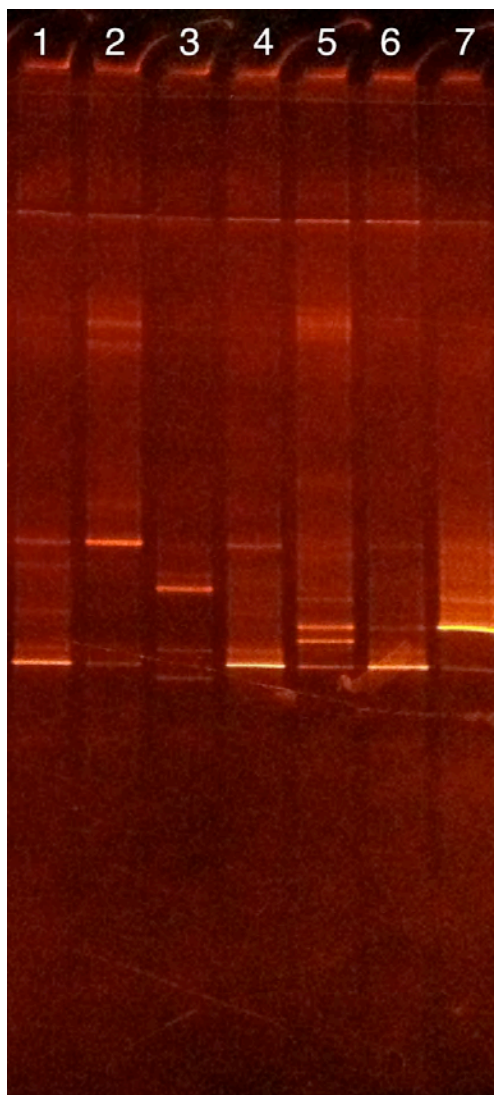


Figure 20. Denaturing gradient gel electrophoresis of the Archaeal community present in the marine algae and seagrasses anaerobic microcosms. The community existent presented a low diversity, however the dominance of the Archea in the microcosms will depend on the aquatic biomass (marine algae or seagrass) utilized. The gradient utilized in the gel presented was of 20 to 60% of bis-acrylamide at 60°C for 12 hours. Lane 1. *Syringodium filiforme*, Lane 2. *Thalassia testudinum* experimental, Lane 3. *Thalassia testudinum* unamended, Lane 4. *Sargassum fluitans* experimental, Lane 5. *Sargassum fluitans* unamended, Lane 6. Experimental inoculum, Lane 7. *M. hungatei*.

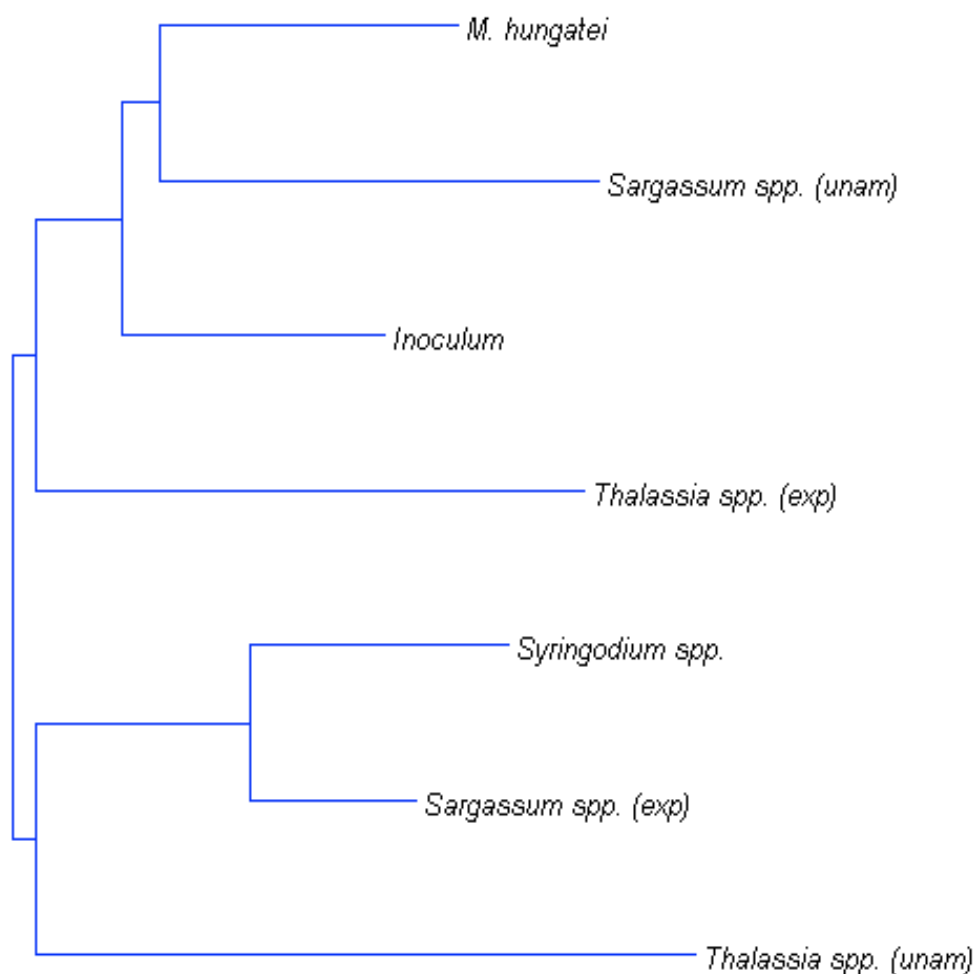


Figure 21. Cluster analysis with Jaccard's similarity of the Archaeal community present in the marine algae and seagrasses anaerobic microcosms. The dendrogram demonstrates that the Archaea present are similar in both the experimental and the microcosms that have no inoculum. However the Archaea dominating in the experimental microcosms will be dependent upon the marine biomass that was being degraded.

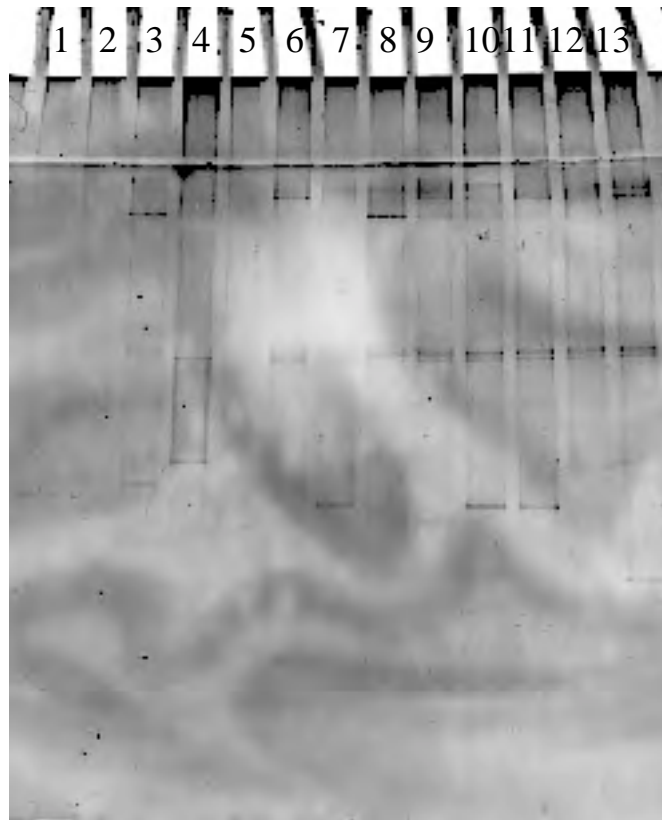


Figure 22. Denaturing gradient gel electrophoresis of the bacterial community present in the anaerobic tropical climbing vines and marine biomass microcosms. The bacterial community present in the microcosms was dominated by a single bacterium, *D. vulgaris*. The gradient of the gel presented was of 20-60% of bis-acrylamide at 60°C for 12 hours. Lane 1. *D. vulgaris*, Lane 2. Empty, Lane 3. Experimental inoculum, Lane 4. *Sargassum fluitans* experimental, Lane 5. Unidentified marine algae experimental, Lane 6. *Gracilaria* sp. experimental, Lane 7. *Thalassia testudinum* unamended, Lane 8. *Syringodium filiforme* experimental, Lane 9. *Desmodium incanum* unamended, Lane 10. *Epipremnum pinnatum* experimental, Lane 11. *Dioscorea bulbifera* experimental, Lane 12. *Cayaponia racemosa* experimental, Lane 13. *Cissus verticillata* experimental.

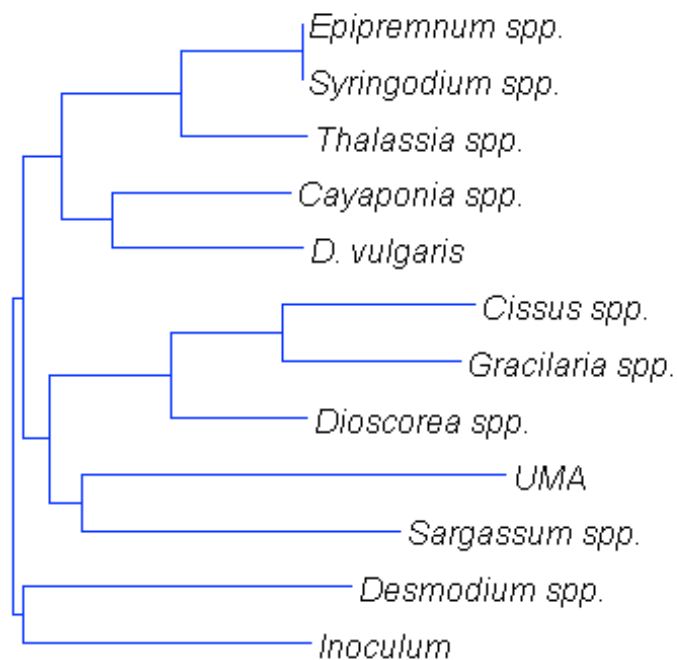


Figure 23. Cluster analysis with Jaccard's similarity of the bacterial community present in the tropical climbing vines anaerobic microcosms. The dendrogram demonstrated that the experimental microcosms were generally grouped within the same cluster, while the unamended microcosms were also in the same cluster.

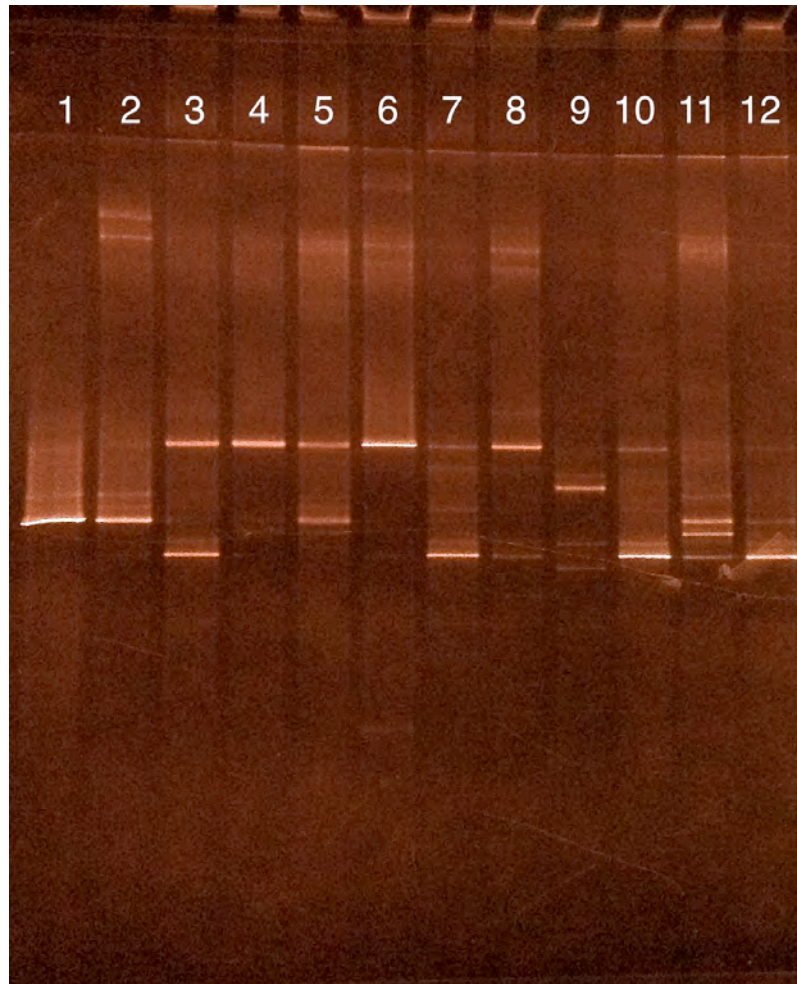


Figure 24. Denaturing gradient gel electrophoresis of the archaeal community present in the tropical climbing vines anaerobic microcosms. The archaeal community present in the microcosms presented a high diversity, however the dominance of the archaea (intensity of the band) will be distinctive to each of the microcosms analyzed. The gradient of the gel presented was of 20-60% of bis-acrylamide at 60°C for 12 hours. Lane 1. *M. hungatei*, Lane 2. *Cissus verticilata* experimental, Lane 3., *Cayaponia racemosa* experimental, Lane 4. *Dioscorea bulbifera* experimental, Lane 5. *Epipremnum pinnatum* experimental, Lane 6. *Desmodium incanum* experimental, Lane 7. *Syringodium filiforme* experimental, Lane 8. *Thalassia testudinum* experimental. Lane 9. *Thalassia testudinum* unamended, Lane 10. *Sargassum fluitans* experimental, Lane 11, *Sargassum fluitans* unamended, Lane 12. Experimental inoculum, Lane 13. *M. hungatei*.

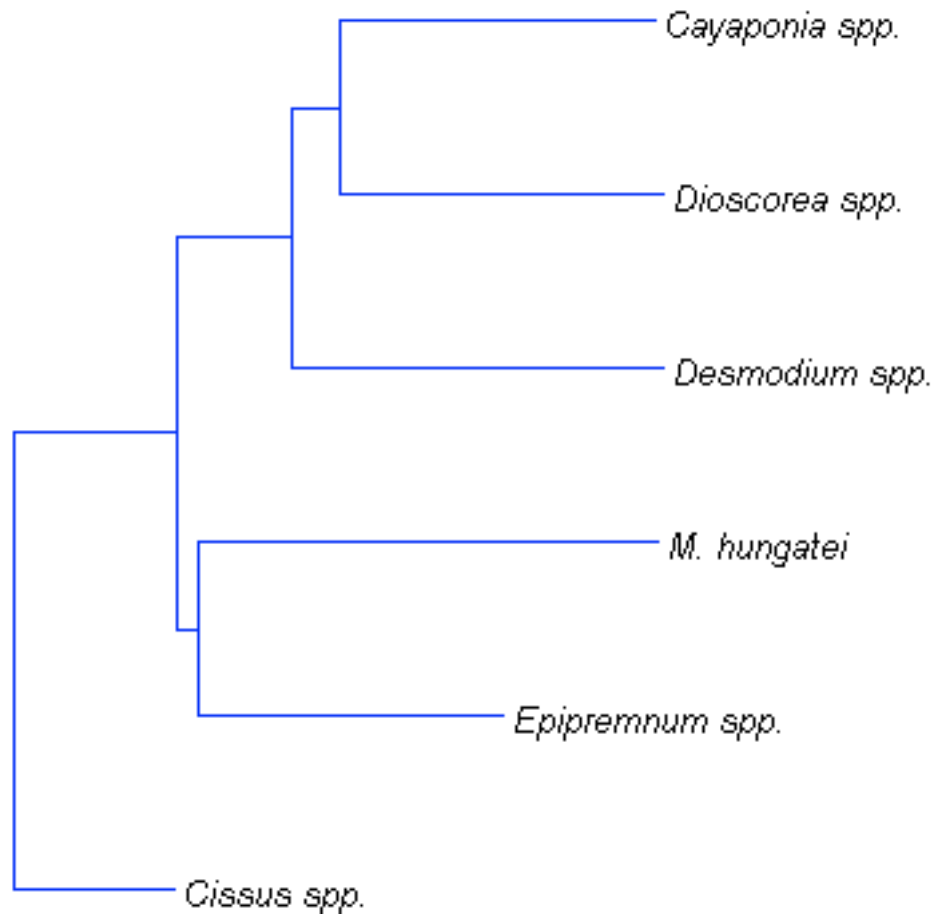


Figure 25. Cluster analysis with Jaccard similarity of the archaeal community present in the tropical climbing vines anaerobic microcosms. The dendrogram demonstrated re is no distinction between the communities present in most of the microcosms, except for the community that was present in *Cissus verticilata*. Its community is unique and separate from the rest of the microcosms.

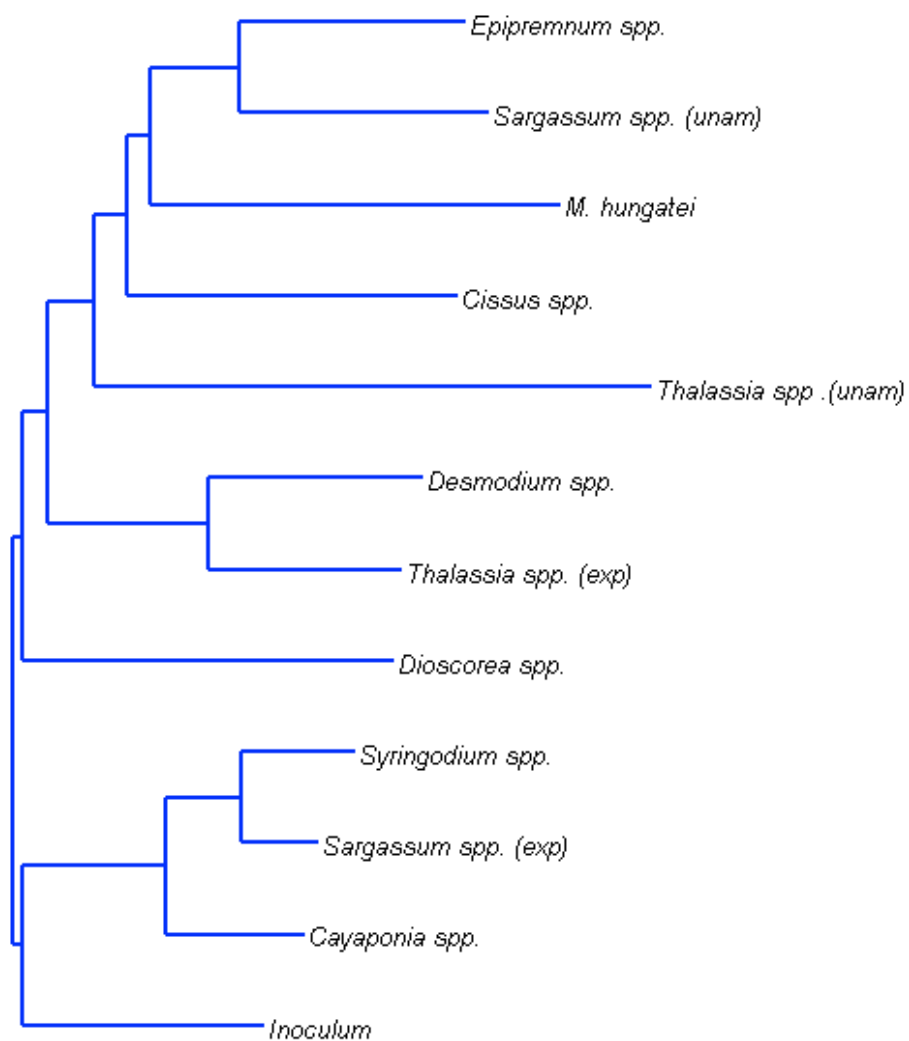


Figure 26. Cluster analysis with Jaccard similarity of the archaeal community present in both the tropical climbing vines and marine biomass microcosms. The dendrogram demonstrate that the community present in both the terrestrial and marine biomasses are similar, since the dominant microorganisms derived from the experimental inoculum.

2.4 Isolation of the anaerobic alginate degraders

2.4.1. Methane production

After monitoring the methane production, of the five natural habitats analyzed, two of those sediments had a higher methane production than the negative control. Methane production in these sediments reached percentages as high as 26% (Mechanic workshop sediment sample) and 12% (Coffee paddy field sample) (Figure 27). Taking in consideration that only these two sediments rendered different methane production, they were diluted to purify the microorganisms present in the sample. In the dilutions of the mechanic workshop sediment samples, the first three dilutions (10^{-1} , 10^{-2} , 10^{-3}) produced a mean methane production of 41.13%, while the highest dilution, 10^{-6} , produce up to 12% of methane (Figure 28). In the Coffee paddy field sediment samples the first two dilutions rendered a mean methane production of 41.37%, while the highest positive dilution, 10^{-4} , produced up to 8.54% (Figure 29).

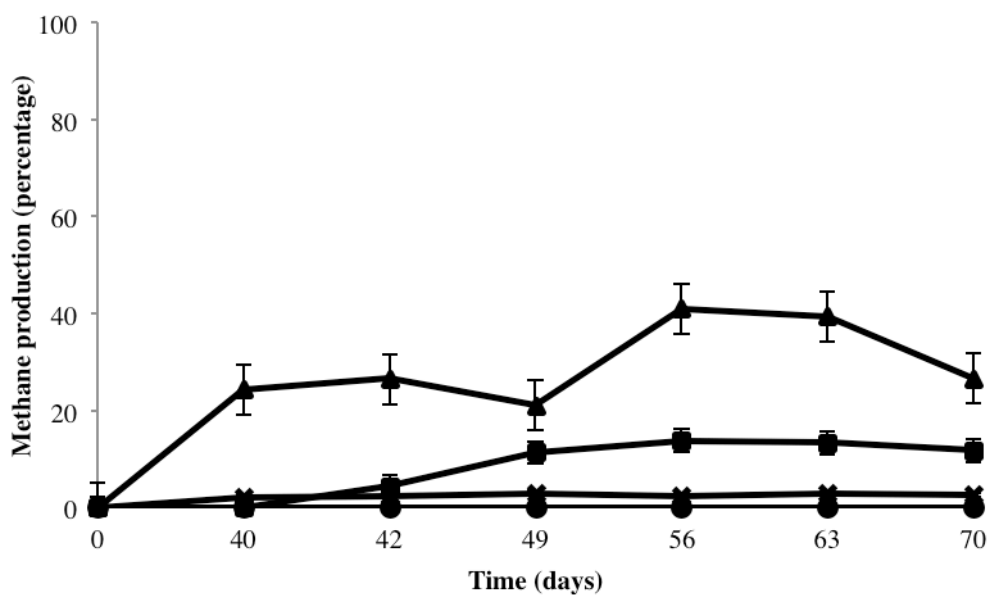


Figure 27. Methane production from five different natural habitats based on the anaerobic degradation of an alginate medium (1%). Based on standard error two natural habitats sediments, Oxidation pond from a Coffee paddy field (■) and a mechanical workshop sediment (▲), produced higher quantity of methane than the other sediments analyzed, Ruminant fluid (◆), Luchetti reservoir (×) and Guánica Dry Forest sediment (●).

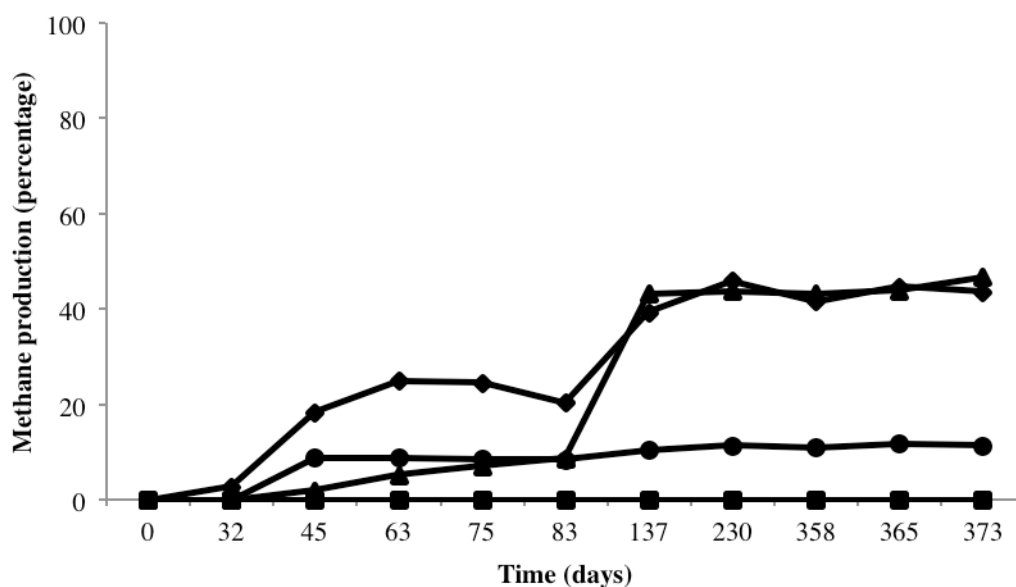


Figure 28. Methane production in the mechanic workshop sediment samples dilutions degrading alginate media. The highest positive dilution, 10^{-6} , allowed up to 12% of methane. 10^{-1} (◆), 10^{-3} (▲), 10^{-6} (●) and negative control (■).

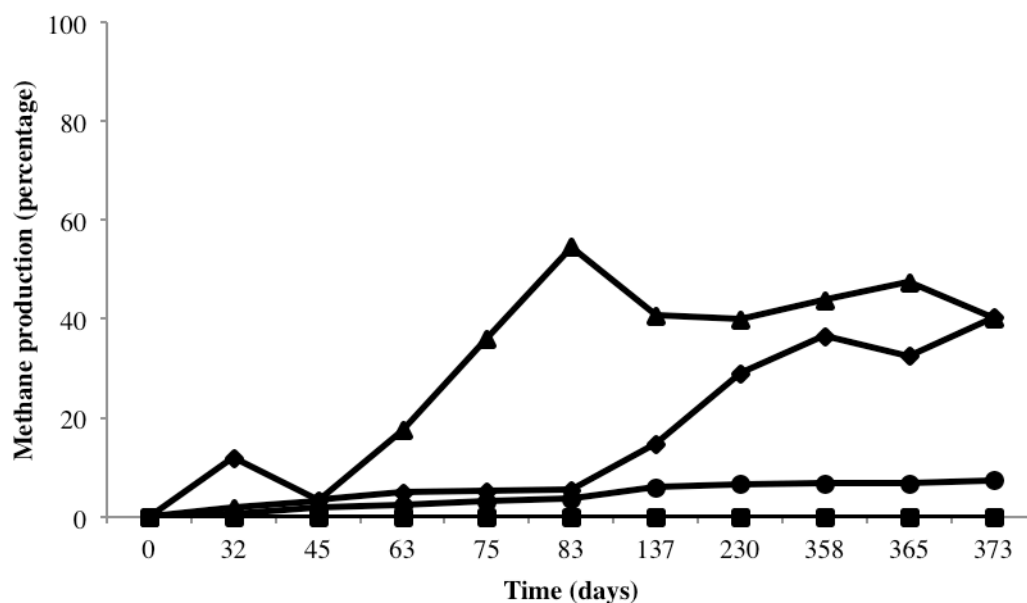


Figure 29. Methane production in the coffee paddy field samples dilutions of alginate medium. The highest positive dilution, 10^{-4} , allowed up to 12% of methane. 10^{-1} (♦), 10^{-2} (▲), 10^{-4} (●), and negative control (■).

2.4.2. Morphological description

To describe the degraders that were purified from both mechanic workshop and oxidation pond of a coffee paddy field sediment samples, phase contrast microscopy was performed. Through the first dilution, both samples revealed a variety of morphologies, which were either unique for each environment or present in both environments (mechanic workshop samples and coffee paddy field samples). In the mechanic workshop sediment samples the microorganisms observed were: a spore former (Figure 30.A), two short bacillus joint (Figure 30.B), and a microorganism that resembles an anaerobic fungus (Figure 30.C). In the coffee paddy field sediment samples, it was also observed the presence of an anaerobic spore former (Figure 31.A), long bacilli (Figure 31.B) and aggregates of microorganisms (Figure 31.C).

After all the samples were highly purified, roll tubes were performed to obtain discrete colonies. In the coffee paddy field sample six colonies were obtained, while in the mechanic workshop samples only three colonies were obtained (data not shown). In the coffee paddy field colonies, six morphologies were observed, where four of those morphologies were shared between colonies (Figure 32 - 36). From the colonies isolated only two colonies (CPF5, CPF6) were pure cultures (based on microscopic examination), while the rest of the colonies (CPF1, CPF2, CPF4) were co-cultures of different microorganisms. The first colony (CPF1) contained two morphologies, a gram-negative spore former previously seen in the first dilutions (Figure 32.A) and a short bacillus (Figure 32.B). The second colony (CPF2) presented three morphologies: a short bacillus (Figure 33.A), a vibrio (Figure 33.B) and a long bacillus (Figure 33.C). These three morphologies were also present in other colonies like CPF1 and CPF4 (Figure 32, 34, 35). The third colony (CPF3) seems to be a pure culture with a long bacillus, which is also present in CPF2 and CPF4 (Figure 34).

The fourth colony (CPF4) contained three morphologies: a vibrio (Figure 35.A), a possible spirochete (Figure 35.B) and a long bacillus (Figure 35.C). These organisms were observed to form aggregates in liquid samples of CPF4, where in comparison to the previous colonies no aggregations were observed (Figure 35.B). Both CPF5 (Figure 36.A) and CPF6 (Figure 36.B) colonies presented a single morphology, a medium sized bacillus, which appeared to be identical in both. In these two colonies, CPF5 and CPF6, it was also observed the aggregation of microorganisms, where the observation of the single bacillus

was infrequent (Figure 36 C, D). Isolated from the mechanic workshop samples, a single morphology was observed in the three colonies, a small bacillus (Figure 37 A, B, C), which was constantly seen in groups or small aggregates (Figure 37 D, E, F). However, in both of the samples (MWS1 and MWS2) it was visible the presence of spores, however no spore former was observed (Figure 37 G, H).

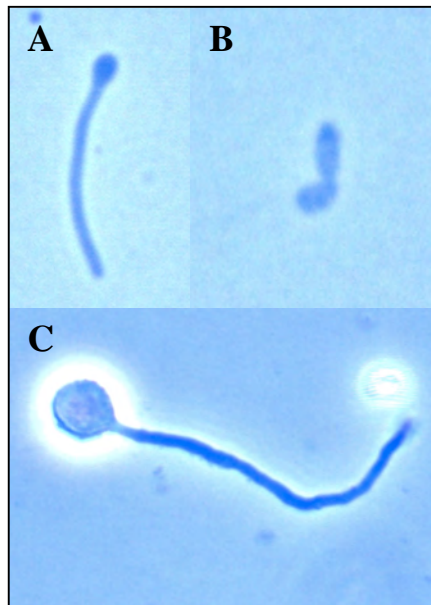


Figure 30. Three morphologies obtained from the mechanic workshop sediment dilutions. A) Spore former, B) two bacillus united, C) a possible anaerobic fungi. Phase contrast microscopy at 1000 X magnification.

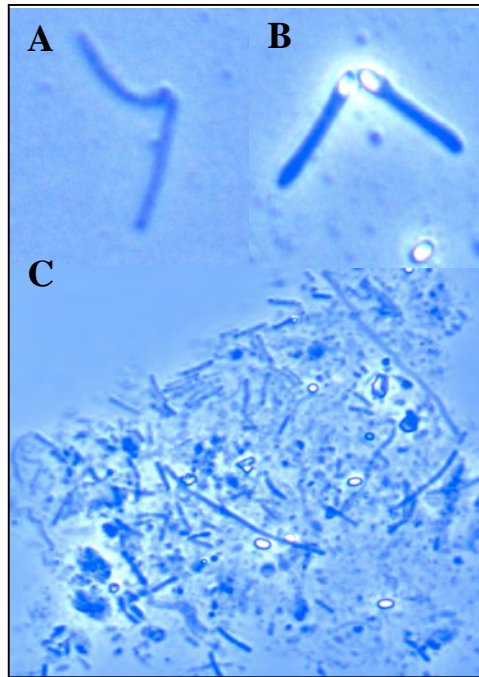


Figure 31. Three morphologies obtained from a coffee paddy field sediment dilutions. A) Two long bacillus, B) Spore former, C) Aggregates of microorganisms. Phase contrast microscopy at 1000 X magnification.

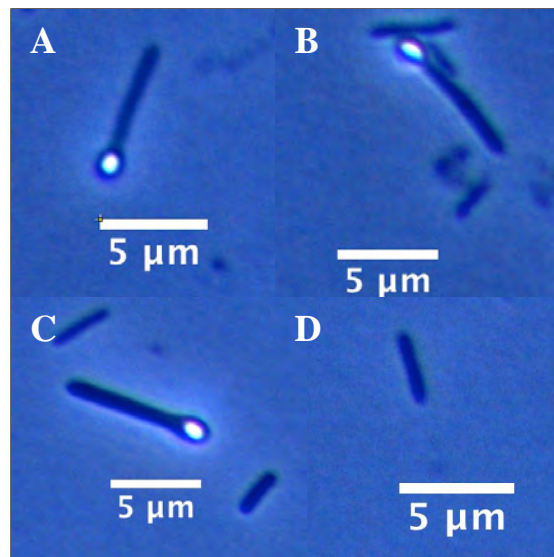


Figure 32. Morphologies of the CPF1 colony isolated from the coffee paddy field sediment. A) Morphology of a spore former, B and C) the presence of both the spore former and bacillus, D) morphology of the small bacilli. Phase contrast microscopy at 1000 X magnification.

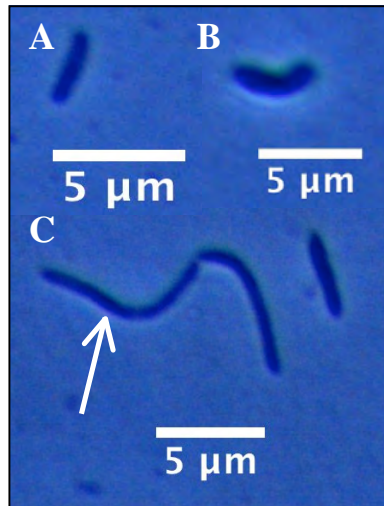


Figure 33. Morphologies of the CPF2 colony isolated from the coffee paddy field sediment. A) Morphology of a small bacillus, B) morphology of a vibrio, C) morphology of a long bacillus with a small bacillus. Phase contrast microscopy at 1000 X magnification.

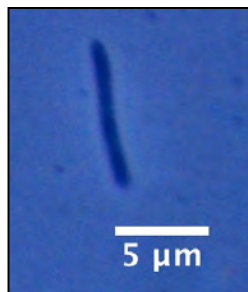


Figure 34. Morphology of the CPF3 colony isolated from the coffee paddy field sediment. Phase contrast microscopy at 1000 X magnification.

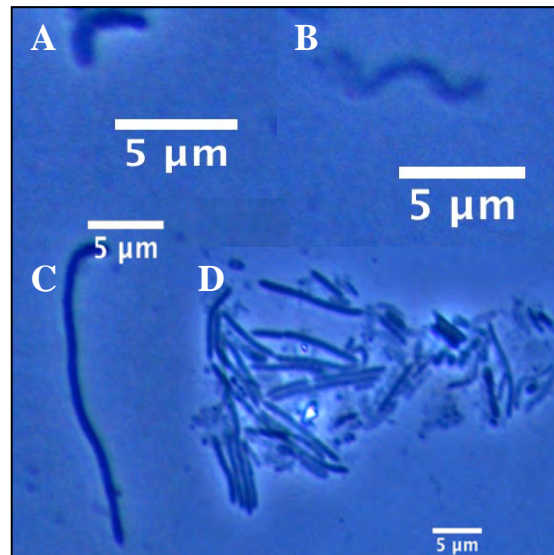


Figure 35. Morphologies of the CPF4 colony isolated from the coffee paddy field sediment. A) Morphology of a vibrio, B) morphology of a possible spirochete, C) morphology of a long bacillus, D) aggregates of the microorganisms. Phase contrast microscopy at 1000 X magnification.

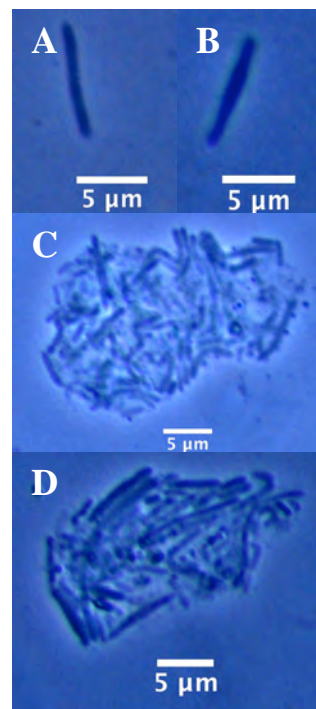


Figure 36. Morphologies of the CPF5 and CPF6 colonies isolated from the coffee paddy field sediment. A) Morphology of a small bacillus from CPF5 colony, B) morphology of a small bacillus from CPF6 colony, C and D) aggregates of microorganisms from CPF5 and CPF6 colonies, respectively. Phase contrast microscopy at 1000 X magnification.

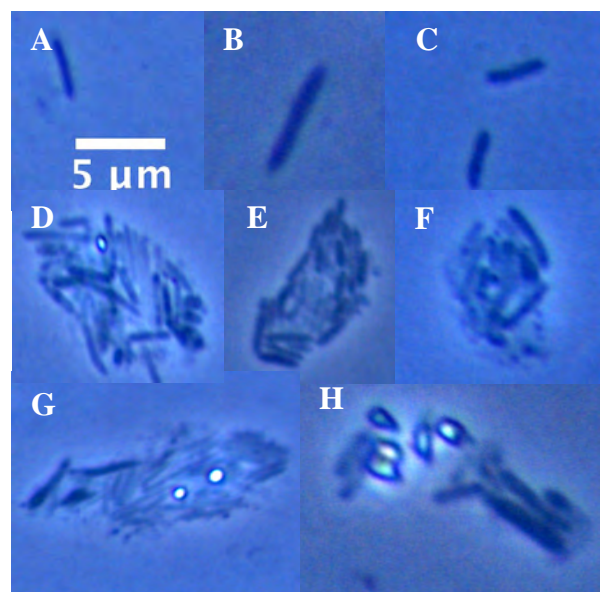


Figure 37. Morphologies of the colonies isolated from the mechanic workshop sediment. A, B, C) Morphology of a small bacillus; A) MWS1 colony, B) MWS2 colony, C) MWS3 colony; D, E, F) Aggregates of microorganisms in MWS1, MWS2, MWS3 colonies; G, H) Spores present in MWS1 and MWS2 colonies. Phase contrast microscopy at 1000 X magnification.

2.4.3. Molecular analysis

After obtaining discrete colonies, DGGE was performed to determine if the colonies were pure cultures or mixed cultures. Following PCR for both Bacterial and Archaeal 16S rDNA gene, the colonies obtained only showed amplification for the Bacterial 16S gene (data not shown). The colonies isolated from both samples, mechanic workshop and coffee paddy field sediment, were bacteria, thus no Archaea was successfully isolated. DGGE for the purified colonies of the Coffee paddy field samples demonstrated that CPF1, CPF2, CPF3 and CPF4 were mixed cultures of two or three bacteria in each colony, whereas CPF5 and CPF6 seemed to be pure cultures (Figure 38). However, only

4 bacteria were present in all the colonies isolated from the coffee paddy field colonies, which meant that certain bacteria were present in more than one isolate. When comparing the bacteria present in the mechanic workshop samples, two of the colonies were pure cultures, while the one was a mixed culture (Figure 39). In mechanic workshop samples, a specific bacterium was present in the three colonies obtained from this sample, which is the same bacterium isolated in both MWS2 and MWS3 colonies. Based on the migration of our positive control, none of the bacteria isolated from the mechanic workshop and coffee paddy field samples seemed to be *S. aciditrophicus* or *D. vulgaris*.

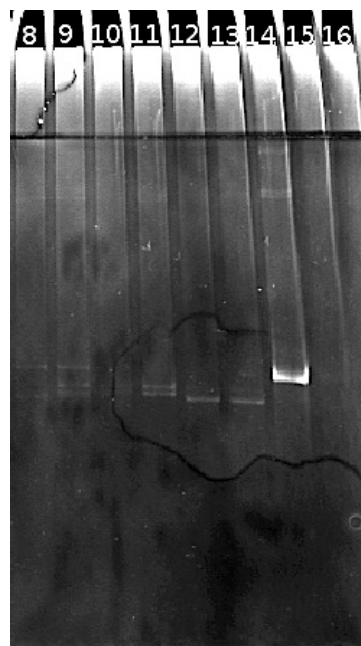


Figure 38. Denaturing gradient gel electrophoresis of the bacterial community present in the colonies isolated from the coffee paddy field sample. The colonies isolated are both pure cultures and mixed cultures, having only two or three bacteria. The gradient of the gel presented was of 30-55% of bis-acrylamide at 60°C for 12 hours. Lane 8. CPF1, Lane 9. CPF2, Lane 10. CPF3, Lane 11. CPF4, Lane 12. CPF5, Lane 13. CPF6, Lane 14. *S. aciditrophicus*, Lane 15. *D. vulgaris*, Lane 16. Empty.

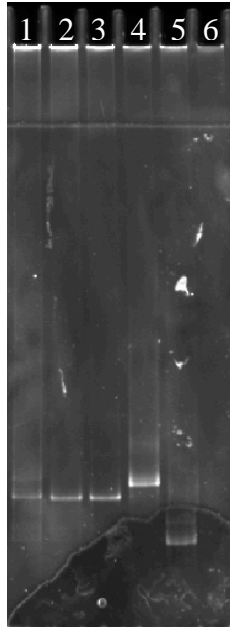


Figure 39. Denaturing gradient gel electrophoresis of the bacterial community present in the colonies isolated from the mechanic workshop sediment sample. The colonies isolated were both pure cultures and mixed cultures, where only one of the colonies has two bacteria. The gradient of the gel presented was of 25-50% of bis-acrylamide at 60°C for 12 hours. Lane 1. MWS1, Lane 2. MWS2, Lane 3. MWS3, Lane 4. *S. aciditrophicus*, Lane 5. *D. vulgaris*, Lane 6. Empty.

Discussion

Tropical climbing vines as potential biomass for the generation of a renewable energy system

Systems of renewable energy have been widely studied to successfully replace the usage of fossil fuels, which are growing scarce. The purpose of the study was to compare and determine which type of photosynthetic biomass (marine algae, seagrasses or tropical climbing vines) serves as a better substrate as primary biomass for the development of an anaerobic reactor. Our initial hypothesis predicted that marine algae would serve as a better substrate for the bioconversion of methane due to the presence of molecules readily degradable in comparison to the terrestrial biomass. The data obtained from the degradation of both aquatic and terrestrial biomass demonstrated that, based on statistical tests, there is a significant difference between the degradation of these biomasses; being that of tropical climbing vines more efficient (Table 5).

Methane production obtained from the climbing vines microcosms rendered up to 50% of methane while in aquatic biomass the highest production of methane reached only 20% (Figure 3). Previous literature has stated that aquatic biomass, specifically marine algae, should be a better substrate in comparison to terrestrial plants due to the lack of molecules like lignin and cellulose (Vergara-Fernández, 2008; Wargacki, 2012). However, based on the results obtained, it is clear that aquatic biomass not always serves as the best substrate, since the methane production in the terrestrial microcosms is almost three times of what was produced in marine algae microcosms. Moreover, when considering the microbial community used to inoculate the microcosms analyzed, 66% of the community present was specialized in the

degradation of marine algae and alginate, while the remaining 33% were cellulose degraders. If more than half of the community was specific towards the degradation of aquatic biomass, and the methane production was still lower than the methane produce by the terrestrial microcosms, it is possible that the cellulose degraders outcompeted the rest of the microbial community.

Cellulose is composed of 1-4 β glucosidal linkages and represents up to 30 to 50% of the dry weight in terrestrial biomass, being one of the most common polymers on earth (Cosgrove, 1997; Carere, 2008). As one of the most common polymers, there is a higher quantity and diversity of cellulose degraders (Bergquist, 1999), and are adapted to degrade efficiently cellulose when available (O'Sullivan, 2005). Research performed by Kato et al., (2004), established that in mixed populations of non-cellulolytic bacteria with cellulolytic bacteria, a positive interaction stimulates the degradation of cellulose by the cellulolytic bacteria. Therefore, the microcosms containing biomasses as the climbing vines and seagrasses, which are composed of molecules like cellulose, hemicellulose and cellobiose (Lynd, 2002; Dawes, 1998) should have the potential to produce higher quantities of methane since they should be almost entirely degraded. Based on the comparison of the climbing vines and the marine biomass (Figure 3), the previous statement is favored since the microcosms with the highest methane formation were the climbing vines and the seagrasses.

If we compare the individual species of marine biomass present in the microcosms, there is no significant different between three species of marine algae and seagrass (*Thalassia testudinum*, *Gracilaria* sp. and the unidentified marine algae). However based on the LSD Fischer Statistical Test there was a significant difference

in methane production between two biomasses: *Sargassum fluitans* (marine algae) and *Syringodium filiforme* (seagrass). These two microcosms represent both the lowest and the highest production of methane, respectively, with a difference in production of 6%. The marine algae *Sargassum fluitans* had been previously analyzed as feedstock for anaerobic digestion and due to the unknown components of fiber and low mannitol concentration they have been qualified as a poor biomass (Bird, 1990). As far as we know, *Syringodium filiforme* has never been study for the development of anaerobic reactors based on the degradation of this biomass. *Syringodium filiforme* is a tropical seagrass with both high growth rates and high mortality, outgrowing and colonizing meadows more quickly than other species of seagrass (Peek, 2012). Nevertheless it has been recorded that it requires high quantities of nutrient and has a low tolerance for natural disturbances (Gallegos, 1994).

In relation to renewable systems based on the usage of marine biomass, like the ones analyzed herein, the biomass is typically grown first on artificial systems known as algae ponds (Posten, 2009). A variety of pond systems have been developed to grow algae; however, one of the most economical methods established up to date is the open pond systems (Resurreccion, 2012). Open ponds systems are large tanks where either macro or micro-algae are grown by injecting CO₂, which is absorbed by algae and incorporated to produce biomass (Huber, 2006). Nevertheless if the creation of a renewable system were to be developed using either marine algae or seagrass, it would depend on the species utilized as primary biomass for the reactor (Ugwu, 2008). For example, *Thalassia testudinum* is another tropical seagrass that in comparison to *Syringodium filiforme* has a low nutrient requirement but a slow growth rate (Davis, 2001). Preceding studies have determined that *Syringodium*

filiforme could produce up to 446.9 g m⁻² of biomass in a year, while *Thalassia testudinum* can produce up to 433.8 g m⁻² of biomass (Gallegos, 1994; Irlandi, 2002). Even though *Thalassia testudinum* grows slower than *Syringodium filiforme* the total biomass produced differs by more or less 13.1 g m⁻², which means that growing *Thalassia testudinum* would produce a more cost effective system. The growth of this biomass would be lower, based on a lower nutrient requirement; even if there is a 3% of methane production lost, as observed in the experimental results.

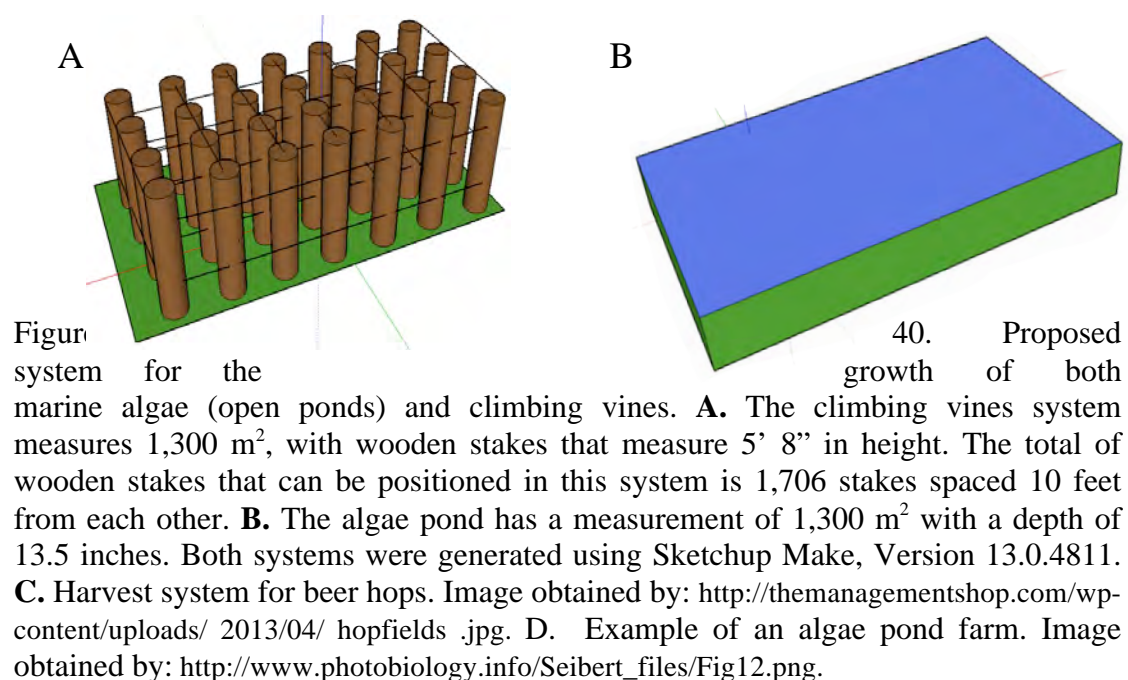
On the contrary, red algae like *Gracilaria* sp. could produce approximately up to 1,800 g m⁻² of biomass per year, while brown algae like *Sargassum fluitans* could produce up to 1,400 g m⁻² of biomass (Gao, 1994). In comparison to seagrasses, the production obtained by marine algae was higher meaning that an even more cost effective system would be created consuming marine algae as primary biomass. Following this further, if a system were to be created to supply a household that consumes an average of 240 kW/h of energy, an average of 1,502.04 kg of biomass would be needed per year (Table 2). In other words if marine algae can produce a total of 1.4 to 1.8 kg m⁻² of biomass per year as stated previously, then an hypothetical open pond system with a diameter of 1,300 m² and a depth of 13.5 inches, would be needed to supply the energy necessary for a year (Gao, 1994). Literature suggests that the depth of the algae pond should not exceed the measurement described (Hadiyanto, 2013), since a pond with a depth higher from the one described would decrease the algal production (Odlare, 2011). Even though it is a viable option, this type of system is commonly used for industrial production of algae biomass, since the space required is too demanding for a regular house.

Besides comparing the marine algae microcosms, the analysis of tropical climbing vines demonstrated that there is no significant difference between four of the species analyzed (*Epipremnum pinnatum*, *Dioscorea bulbifera*, *Desmodium incanum* and *Cayaponia racemosa*) (Figure 2). Nonetheless, based on LSD Fischer Test, there is a significant difference for the degradation of *Cissus verticilata* in anaerobic environments (Table 3). *Cissus verticilata* is a tropical climbing vine, non-woody, that can colonize disturbed areas where sufficient light is available (Acevedo-Rodríguez, 2005). This family is composed of more than 80 species, which have been extensively studied for the generation of medicinal remedies (Pepato, 2003). However, recent studies have described the high concentration of complex molecules like tannins and sulfated products in this species of climbing vine (deOliveira, 2012), which have been proven to be inhibitors of the process of methanogenesis (Mitterer, 2010; Kamra, 2006), providing then a possible explanation into why this biomass did not provide the methane rendition observed by the other vines.

However tropical climbing vines, similarly to the seagrasses, have never been previous analyzed for the creation of an anaerobic reactor to generate renewable energy systems. Climbing vines are classified as lignocellulosic biomass, which have been proposed to substitute biomass related to the food industry for the creation of biofuels, mainly bioethanol. The substitution of this biomass would firstly, eliminate the raise of food prices, and secondly produce an abundant source since they represent up to 50% of the available biomass in the world (Claassen, 1999). Many scientists have determined that the pretreatment, either physical o chemical, of this biomass is necessary so the conversion of the biomass is efficient and cost effective (Yang, 2009). However though the pretreatment may help towards a higher rendition of

methane (Hendricks, 2009), the methane rendition obtained in the microcosms analyzed without pretreatment was extremely efficient, up to 50% of methane. The methane obtained in these microcosms not only proved that pretreatment may not be always necessary, but that they are better biomass than marine algae (Figure 3). However to determine if the tropical climbing vines are in fact more efficient, it is also necessary to determine how cost effective the system that should be created would be in comparison to one developed with marine algae as previously described.

Many scientists have shown that climbing vines have high and rapid growth rates (Carrasco-Urra, 2009), however few of those studies have quantified the biomass production obtained in a period of time. Nevertheless, for the purpose of this analysis, biomass generation was based on the study provided by Sweeney et al. (1975), which determined the growth rate of a selection of species of climbing vines. The growth rate of the climbing vine will depend, as any biomass, on the availability of nutrients and resources; however, based on the previous study, climbing vines can produce up to $293.83 \text{ m}^{-2} \text{ g}$ of biomass in a year. In comparison to the biomass production of marine algae or seagrasses, these plants produce significantly less biomass, still the requirements for their growth would be less than in the marine biomass (Huber, 2006). The growth of marine algae and seagrasses is generally carried out in open ponds and, as established previously, $1,300 \text{ m}^2$ would be needed to generate the biomass necessary to supply the energetic rendition of a household consuming 240 kW/h monthly, rendering these types of systems better suited for industrial purposes than for household purposes. Taking in consideration that tropical climbing vines do not have to be grown on an aqueous environment, a different model for their growth is suggested as shown in Figure 40.



In comparison to marine algae, tropical climbing vines have a few advantages that could be exploited for their growth in these artificial systems. Climbing vines have the ability to grow either vertical or horizontally and the main nutrient required for their growth is CO₂, besides minerals, which can be reduced from the atmosphere by sunlight and water (Granados, 2002). If we then grow the climbing vines the same way as the algae pond previously described (1,300 m² with a depth of 13.5 cm) we could generate an approximate of 754.09 kg of biomass, much less than the marine algae. However if we then take into consideration the dimensions of the system generated in Figure 40, the system generates four times the biomass produced by algae ponds. Based on the previous calculations, then a total biomass of 3,016.36 kg can be generated, which could last for almost four years and six months for an energetic consumption of 240 kW/h every month. After stating the previous facts, it is

clear that the tropical climbing vines provide a more cost effective and efficient system than the marine algae. Moreover the system that could be generated with the use of tropical climbing vines can be useful for both industrial and household purposes, since the system can be reduced to fit in a house and still provide the biomass required to supply the energetic necessity.

Even though that using climbing vines to create the renewable energy system proposed, provides a more complete system than the marine biomass, there are some details that still need to be studied. In general terms, the marine biomass can take an average of 67 days to provide the methane rendition that can be obtained from their degradation (Figure 1). On the other hand, the time required to degrade the climbing vines will vary among species, and more importantly it is lengthier than the marine biomass. It is probable that the period it takes for the microbial community to degraded the biomass and stabilize the methane production may be correlated to the adaptation phase of the different microcosms. In terms of adaptation, marine algae and seagrasses microcosms presented an adaptation period of 8 days before the methane production was detected (Figure 1). Whereas in the tropical climbing vines the adaptation periods can be divided in three stages: 8, 22 and 50 days (Figure 2). In this case, to determine why climbing vines require more time for its degradation, it is important to describe the molecule that may be present in the marine biomass (marine algae and seagrasses) and the tropical climbing vines (Appendix 1).

In comparison to the tropical climbing vines, marine algae and seagrasses have less content of complex molecules like lignin and cellulose and a higher content of degradable sugars and carbohydrates (Dawes, 1998). Bearing in mind that the

described structures are easily converted to intermediaries (Tahuer, 1998), we suggest that these molecules are the first to be degraded and converted for methane production. Since the cellulose content in these microcosms is low, the cellulose degraders will start the slow consumption of these molecules liberating intermediaries that will be converted eventually to methane. However since the part of the microbial community will be degrading molecules that are more accessible (sugars) first, there will be an accumulation of organic acids during the consumption of these sugars, which will be representative of the degradation of cellulose as seen in Figures 4, 5, and 6. Contrary to marine algae, in climbing vines more than half of the molecules present are cellulose, hemicellulose and lignin (Carroll, 2009). Due to the complexity of these molecules, the microbial community will require more time to degrade these molecules and convert them to the intermediaries necessary for methane production (Leschine, 1995). Moreover some scientist argue that in anaerobic environments lignin is not degraded, which means that not only will the carbon present in the molecule will be sequestered in this polymer and not converted to methane but the effort of the microbial population to degraded it will be lost (Kirk, 1987; Tuomela, 2000). For which the microcosms containing climbing vines present a longer adaptation period in comparison to the marine algae microcosms. Like any system, pretreating the biomass as previously mentioned, either by physical or chemical reactions could benefit in the optimization of this process. The pretreatment of the biomass can help in reducing both the adaptation and degradation period and help increase the methane production (Taherzadeh, 2008). In summary, after comparing methane rendition, cost-effectiveness, and optimization processes, the system, which will be more efficient and effective, is an anaerobic reactor digesting climbing vines.

Microbial community

Furthermore the second objective of the study was to provide an insight into which microorganisms may have been present in the microcosms and dominated in the degradation of marine algae, seagrass and tropical climbing vines. When determining the diversity present in the samples it was based on the quantity of bands present in each sample analyzed, since each band is representative of a specific microorganism. On the other hand, the dominance of the microorganisms in each sample was determined by the intensity of the band. In terms of the bacterial community present in the marine algae and seagrasses microcosms there appeared to be a convergence in the community to a few similar microorganisms. DGGE data suggests that the bacterium that appeared to dominate in all species of marine algae and seagrasses is *D. vulgaris*. It isn't uncommon to observe microorganisms like *D. vulgaris* present in environments associated with marine biomass since it is a sulfate-reducing bacterium, an event common in oceans (Oremland, 1982). However, it is clear that there appeared to be a preference towards the selection of this bacterium to dominate on all the species analyzed over other bacteria. A possible explanation for the dominance and the selection of this bacterium is that sulfate-reducing bacteria, in presence of sulfate, have a higher affinity towards the precursors of methane production than methanogens, favoring energetically this reaction (Isa, 1986). Taking in consideration that in some microcosms there was in fact sulfate reduction, then it could be possible that this bacterium was then being favored by the conditions present in the microcosms. Furthermore, it is possible that these microorganisms used acetate while reducing sulfate (Brandis, 1981). Some species of *Desulfovibrio*, like *D. vulgaris* are able to grow on acetate, H₂ and CO₂; utilizing acetate and CO₂ to obtain

their carbon source and the energy from H₂ (Pankhania, 1986; Bryant, 1977). In anaerobic environments, the consumption of one mol of acetate will provide an equimolar quantity of methane: $\text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CH}_4 + \text{CO}_2$ (Schinck, 2006). When observing then the production and consumption of acetate in the marine biomass microcosms it is clear that it is not a coupled event, since in some cases for every 4 moles of acetate one mol of methane is produced. Therefore it is possible that *D. vulgaris* is responsible for degrading 75% of the acetate while the remaining 25% is degraded by a methanogenic bacterium.

On the other hand, the archaeal community demonstrates that though there is a low diversity there appears to be a selection towards the dominating microorganism. In some cases this methanogen will derive from the community that was present in the biomass before inoculation, meaning it survived desiccation and high temperatures for 24 hours, and in others it will come from the experimental inoculum. Although the archaeal community in some experimental and unamended (not inoculated) microcosms is similar, the dominating archaea will differ based on the marine biomass present. This could be an indication that there is no selection towards the bacterium selected but there is for the methanogenic archaea, since the bacterium is favored by the conditions present in the microcosms. In both *Thalassia testudinum* and *Sargassum fluitans* microcosms, the methanogenic archaea present are different but the dominant bacterium in both biomasses is possibly *D. vulgaris*. It has been described that in anaerobic environments bacteria may select a specific methanogenic archaea as partner in order to degrade effectively the substrate present (Schink, 1997; Traore, 1983), however no study has determined what drives these microorganisms towards the selectivity of their partners.

The tropical climbing vines microcosms demonstrated that there was a higher diversity of bacteria in comparison to the marine biomass microcosms. In the marine biomass microcosms in addition to a low diversity, the bacterium that appeared to dominate was, in general, the same in every experimental microcosm. Though in tropical climbing vines there is a higher diversity than the marine biomass, there are a few bacteria that dominate in the experimental microcosms, exhibiting the same behavior as the marine biomass. DGGE data demonstrates the presence of two bands dominating in the majority of the experimental microcosms, being one of them *D. vulgaris*. When analyzing the possible functions of these bacteria in the terrestrial microcosms, based on the production and degradation of fatty acids, DGGE data limits the possible explanation of the work carried out by the microbial community present. Fatty acids are accumulated in a higher concentration and for a longer period than the ones produce in the marine biomass microcosms. For which if we were to describe the possible roles of the bacteria present there should be a minimum of at least three microorganisms present. However in some microcosms only one or two bands are observed in the DGGE gel, for example in *Dioscorea bulbifera* microcosms. In *Dioscorea bulbifera* microcosm lactate is initially present in high concentrations (109mM) and degraded in a period of 8 days (Figure 11). The consumption of one mol of lactate in anoxic conditions will produce an equimolar concentration of acetate: $\text{CH}_3\text{CH}_2\text{OHCOO}^- + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{HCO}^- + \text{H}^+ + 2\text{H}_2$ (McInerney, 1981). However the concentration of acetate produce in this microcosm is not coupled to the degradation of lactate for which it is then possible that the bacteria may be converting the lactate to another product that is not reflected in our analysis. It is expected then a minimum of at least two or three bacteria should be

present in this microcosm; where one is consuming lactate, another producing acetate at a lower rate and another consuming other fatty acids produces like butyrate or propionate. However, as previously stated, only one bacterium was observed in DGGE, for which the explanation of what this bacteria are doing is limited by molecular analysis. This behavior is observed in the majority of the terrestrial microcosms, where in the experimental samples the highest number of bands (representative of the bacteria present) is not correlated to the number of bacteria that should be present in the sample.

Nonetheless, when analyzing the dominance of the bacteria in the samples it is then suggested that in both environment (marine biomass and terrestrial microcosms) there appear to be certain conditions that favor a specific bacterium. It is possible that the conditions that favoring the marine biomass microcosms are events such as sulfate reduction, which is observed in both marine algae and seagrasses (Westrich, 1984). While in the tropical climbing vines microcosms, the complex structures available like cellulose and lignin, could be favoring the selection of certain bacteria. In comparison to tropical climbing vines, both marine algae and seagrass exist in a saline environment containing up to 3% of salt (Touchette, 2007). However, when terrestrial plants are exposed to salt or saline environment it limits their ability to absorb nutrients and water, causing in some cases death (Munn, 2002). It is then possible that in marine biomass microcosms, the diversity of the microbial community is being limited by salinity, since the salt may still be present in the biomass after being dried. Since the tropical climbing vines will not face this limitation, it will reflect a higher diversity of the community in comparison to the marine biomass.

In contrast to the bacterial community, the archaeal community demonstrates distinctive domination in all experimental microcosms. For which in both, the marine biomass and tropical climbing vines, the archaea dominating will be specific and different in each biomass that is being analyzed. Nonetheless in the same way that the bacterial community is more diverse in the terrestrial microcosms, the archaeal community is also a more diverse community. In anaerobic environments, methanogenic archaea are categorized by the type of substrate they utilize: hydrogenotrophic methanogens (H_2/CO_2) or acetoclastic methanogens (acetate) (Angel, 2011). In the terrestrial biomass microcosms high quantities of acetate are produce and degraded throughout the experimental time, for which it is then possible that the types of methanogens present in the microcosms are acetoclastic methanogens. Since the concentration of acetate in the terrestrial biomass microcosms were, in some cases, almost four times higher that in the marine biomass microcosms, the availability of this intermediary may then permit for a more diverse group of methanogens. In conclusion, when comparing the total community, for both bacteria and archaeal community, there appears to be a higher diversity in the terrestrial biomass. Nevertheless the behavior observed in the community is similar in both terrestrial and marine biomass, where conditions favor the dominance of a bacterium while the dominance of the archaea will be distinctive for each biomass analyzed.

Bacteria capable of degrading alginate in anaerobic environments

The last aim of the study was to isolate and describe microorganisms from different natural habitats with the capacity to degrade alginate in anaerobic conditions. As stated in our previous objective, one of our purposes was to analyze whether the degradation of marine biomass was efficient for the creation of a renewable energy system. Though in comparison to terrestrial biomass they are not the ideal biomass, the process could be optimized to generate a higher quantity of methane. One of the stages that could be optimized in this system could be the addition of microorganisms that can degrade specific components of the algae like alginate. Some species of marine algae contain alginate in their cell wall, a complex polymer composed of 1,4- β -D-mannuronic acid and α -L-guluronic acid (Pawar, 2012), which can represent up to 40% of the dry weight of this biomass (Davis, 2003). In this last part of our study five natural sediments were selected and enriched in an anaerobic media containing 1% of alginate as sole source of carbon and energy, to determine if there were microorganisms that had the ability to degrade alginate and convert it to methane. However, only two of the five sediments analyzed contained microorganisms that produced more methane than the negative control: Mechanic workshop sample and Coffee paddy field sample. Based on methane production the sediment with the highest methane production was the Mechanic workshop sediment, producing up to 26% of methane (Figure 29), while the Coffee paddy field produce half of the methane in comparison to the first sediment. The production of methane is indicative of microorganisms that can degrade the alginate and produce the intermediaries necessary for methane production. An interesting fact from the sediments that produce a positive production is that these two environments have no

relation to the components that may be present in a marine environment. The only relation that could be describe in one of the samples, specifically the Mechanic workshop sediment sample, is the existence of oil, petroleum and diesel, which are components that might be possibly present in marine environments due to human contamination (Ekhaïse, 2011).

After selecting those environments with a high production of methane, the samples were diluted to purify the responsables microorganisms. The organisms enriched in those dilutions were observed by phase contrast microscopy. In both environments we observed a common morphology, a gram-negative spore former, suggesting a convergence in the community. Literature proposes that convergence in the community may be driven by factors such as competition, where the fittest microorganisms may be selected over time (Massol-Deyá, 1997; Jiang, 2006). It is then possible that this spore former may outcompete other microorganisms in the degradation of alginate or its intermediaries, which could favor its selection in both environments. In order to continue the purification process a second serial dilution was performed followed by roll tubes to obtain discrete colonies. A total of nine colonies were obtained, six from the Coffee paddy field samples and three from the Mechanic workshop samples. In terms of the microorganisms present in the coffee paddy field, the gram-negative spore former seen in the first dilution was isolated. However the colony from which this microorganism was obtained was not pure, meaning that this colony is composed of more than one microorganism. In this case this colony presented two different morphologies, the spore former and a bacillus (Figure 34), and based on DGGE analysis only two bacteria are present (Figure 40).

This means that if in fact there are only two bacteria present like DGGE suggest, then the only bacteria present could be the two morphologies observed.

In terms of the spore former, only a few anaerobic gram-negative spore formers have been described in the literature. Based on the morphological description and substrate utilization, this bacteria is similar to the previously describe gram-negative spore former *Acetonema longum*. Kane et al. (1991), described this microorganism as a strict anaerobe, measuring 6 μm and capable of fermenting sugars converting them to intermediaries such as acetate, propionate, butyrate, succinate and CO_2 . Moreover, when grown on solid media the colony have an appearance of uneven surfaces with a translucent white color. When comparing then the morphological description of both microorganisms we observe that they share the formation of a terminal spore, measure 6 μm , develop similar colonies and may possibly ferment sugars as glucose, fructose and mannose (products of the fermentation of alginate). It is then possible that the microorganism isolated in the Coffee paddy field sample could be *A. longum*. However to confirm that in fact the microorganism isolated is *A. longum* further molecular analysis is required; though there are a limited number of gram-negative spore former described in the literature, and due to their dissimilarity in their morphological description it is possible that the bacteria isolated is the previous mentioned.

Another morphology observed in the colonies isolated from the Coffee paddy field sediment is a vibrio (Figure 35). In general many *Vibrio spp.* have been isolated from marine environments because of their association with marine animals (Rameshkumar, 2010). Moreover some described species of *Vibrio spp.* have been

documented as being capable of fermenting sugars in anaerobic environments (Shieh, 2003; Shieh, 2000). Some of the sugars in which they are described as capable of growing are glucose, mannose, mannitol and sucrose (Xu, 2009). To be able to degrade alginate it is necessary to possess alginate lyase, which cleaves the glycosidic bonds of the polymer (Matsushima, 2010). When these glycosidic bonds are eliminated they liberate oligosaccharides that are then converted to sugars, which other microorganisms can use as carbon source (Kim, 2011). Previous studies have isolated alginate lyases from different species of *Vibrio spp.*, which means that these microorganisms possess the machinery necessary to degrade alginate (Uchimura, 2010). When these microorganisms break down the glycosidic bonds they liberate the intermediaries needed by other microorganisms to produce methane. It is then possible that this *Vibrio* may be secreting lyases into the environment, breaking the glycosidic bonds which in consequent could release the sugars that other microorganisms, like the spore-former, could require.

An additional morphology observed in the coffee paddy field colonies is what appears to be a spirochete (Figure 37). Magot et al., (1997), previously described *Spirochaeta smaragdinae*, an anaerobic spirochete, measuring 6 μm , with an optimum growth at 37°C and the ability to grow on mannose and xylose, while producing translucent colonies with even edges. In terms of comparing the morphological description of our microorganism with the description provide by Magot et al., both microorganisms have a length of 6 μm and possibly growing on sugars such a mannose, which can be produced by the degradation of alginate. However, though the morphological description is similar, again, it is necessary molecular analysis to confirm whether the microorganism observed is *S. smaragdinae* or another species of

spirochete. On the other hand various colonies had the presence of a microorganism with the morphology of a bacillus. However, though the morphology in general is the same, in some colonies the width and length of the bacteria differ. Previous literature has described microorganisms with similar morphological description that, in anaerobic environment, can degrade alginate from marine algae (Tang, 2008).

In conclusion, based on the previous statements, the microorganisms isolated from the environments analyzed, the mechanic workshop and coffee paddy field, are microorganisms that may have been previously described in the literature. We hypothesize that in samples like the coffee paddy field, if the microorganisms present are indeed the ones suggested, it is then possible that the *Vibrio* (if in fact posses the alginate lyases) break the glycosidic bonds, releasing sugars that species such as the spirochete and spore-former will ferment to intermediaries such as acetate, succinate, and propionate, where in the presence of a methanogenic archaea will be converted to methane. To establish if the degradation of alginate occurs in this manner it is necessary to perform other studies to confirm their roles in these microcosms. However, since the microorganisms isolated could not been obtained in pure cultures, it is then possible that the fermentation of alginate is accomplished by a co-culture of the microorganisms described, which could affirm what we hypothesize. Though to confirm that the microorganisms isolated are the microorganisms suggested, there is a need to sequence the DNA from these cultures. Nonetheless, it is interesting to observe that many of the organisms described, where originally isolated from marine environments, where in our study they were isolated from places that are not related to marine environments.

Conclusions

- When comparing the use of marine algae, seagrasses or tropical climbing vines as primary biomass for anaerobic degradation, tropical climbing vines were a more biodegradable biomass, having a higher rendition of methane; therefore our hypothesis is rejected.
- When comparing between the species of marine algae or seagrasses there appears to be a significant difference between the degradation of *Sargassum fluitans* and *Syringodium filiforme*, being the last one a better biomass.
- The tropical climbing vines demonstrated that there is a significant difference in the biodegradation of *Cissus verticilata* versus *Desmodium incanum* and *Dioscorea bulbifera*, proving that *Cissus verticilata* was the least biodegradable biomass.
- In terms of the microbial community analyzed it was observed there was a dominant bacterium, possibly *Desulfovibrio vulgaris*, for both the marine and terrestrial biomass, which may be favored by conditions that are related to the type of biomass present in the microcosms. However since the dominant archaea was different in every biomass analyzed it appears there is a selection of this type of microorganism in the different microcosms.
- Based on morphological description, the isolation of anaerobic alginate degraders appears to be microorganisms that may have been previously described in the literature, such as *Acetonema longum*, though none have been described to possess this characteristic.

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Appendix

Appendix A: Carbon component of the biomass analyzed towards methane production based on the anaerobic degradation of different species of marine algae, seagrass and tropical climbing vines.

	Terrestrial biomass	Marine biomass	
	Climbing vines	Marine algae	Seagrass
Cellulose	+	+	+
Lignin	+		+
Hemicellulose	+		+
Pectin & Xyloglucans	+		
Glucomannan	+		
Xylan	+		
Protein		+	+
Carbohydrates			+
Mannitol		+	
Alginate		+	
Fucoidans		+	
Glycogen		+	
Galactans		+	

The information presented is obtained from literature previously published (Mellerowicz, 2001; Carroll, 2009; Acevedo-Rodríguez, 2005; Barsanti, 2006; Davis, 2003; Dawes, 1998; Fleurence, 1999; Moen, 1997; Vreeland, 2000).

Appendix B: Energy conversion based on methane production.

To determine the energetic rendition that can be obtained based on the anaerobic degradation of the biomasses analyzed, it is necessary to convert the methane percentage to grams of methane.

Exam. *Desmodium incanum* produced 50.23% of methane.

50.23% / 100 of the total gas mass = 0.5023 g of CH₄

0.5023 g = 0.0005023 kg of CH₄

To obtain the kilo Joules of that microcosm we used the following equation:

$$\mathbf{0.0005023\text{ kg} \quad \times \quad 55,530\text{ kJ/kg of CH}_4 = 27.89\text{ kJ of CH}_4}$$

After calculating the kilo Joules, we convert the value to Kw/h with the following conversion:

$$\mathbf{1\text{ kJ/h} = 0.0003\text{ kW/h} \quad 27.89\text{ kJ} \quad \times \quad 0.0003\text{ kW/h} = 0.00836\text{ kW/h}}$$

We then convert kW/h to W/h, with the following equation:

$$\mathbf{1\text{ kW/h} = 1000\text{ W/h} \quad 0.00836 \quad \times \quad 1000\text{ W/h} = 8.36\text{ W/h}}$$